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THE NUTRITION OF THE COTTON RAT (SIGMONDON HISPIDUS HISPIDUS)¹

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ONE FIGURE

(Received for publication August 2, 1943)

Armstrong ('39) has reported that poliomyelitis can be transmitted to the cotton rat and Jungeblut ('40) found that these animals were susceptible to diphtheria infection and intoxication. Clark and Jungeblut ('40) concluded that the cotton rat does not require vitamin C. The apparent increasing importance of this animal for experimental work seemed therefore to justify a further study of its nutritional requirements.

Twelve pairs of animals were obtained from Dr. B. J. Meyer of the Zoology department in June 1942. They have been maintained on the Steenbock stock ration no. 15 and have produced approximately 400 offspring in 1 year. Meyer ('42) has made extensive studies on the breeding conditions, the reproduction and life cycle as well as the endocrine systems of the cotton rat. The young are born with a coat of fur; their eyes open in 24 hours, and they can be weaned at the age of 10 days. We have found, however, that animals weaned at the age of

¹ Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by a grant from the National Live Stock and Meat Board made through the National Research Council. We are indebted to Merck and Company, Rahway, New Jersey, for supplies of thiamine, nicotinic acid, riboflavin and pyridoxine; to Abbott Laboratories, North Chicago, Illinois, for the haliver oil; and to Wilson and Company, Chicago, Illinois, for the liver extracts.

3 weeks and weighing between 20 and 25 gm. give the most satisfactory experimental results. A growth period of 6 weeks was used for all of the studies reported in this paper. At the end of this time normal animals weighed between 60 and 70 gm., while the adult rats weighed 140–160 gm. Growth of male and female animals proceeds at approximately the same rate (fig. 1) although the female is usually slightly smaller.

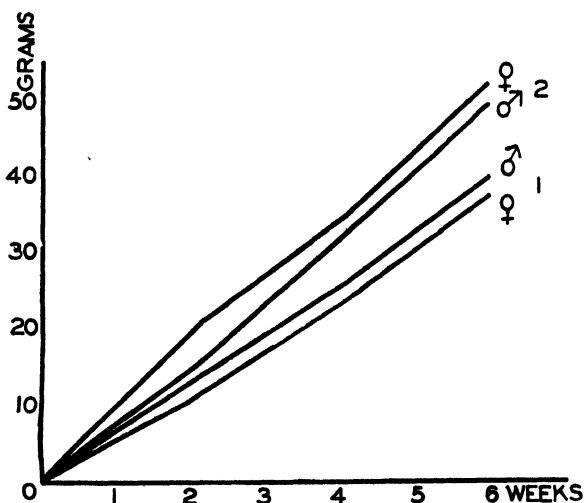


Fig. 1 Growth curves of male and female cotton rats. (1) on ration 801; (2) ration 801 plus 4% 1:20 extract.

EXPERIMENTAL

Since the cotton rat grew well and reproduced on the stock ration, animals on this ration were used as controls for further dietary studies. Experiments were first devised to determine whether the young rat could survive on the synthetic ration which supports growth and reproduction of the white rat. This ration (800) consists of casein,² 18%; sucrose, 73%; salts IV,³ 4%; corn oil,⁴ 5%; thiamine, 250 µg.; riboflavin, 300 µg.; nicotinic acid, 2.5 mg., pyridoxine, 250 µg.; pantothenic acid, 2 mg.; and choline, 100 mg. per 100 gm. of ration. Two drops

² Labeco from Borden and Company, New York City.

³ Phillips and Hart (J. Biol. Chem., vol. 109, p. 657, 1935).

⁴ Mazola.

of halibut liver oil were fed bi-weekly. Three groups of six animals each were placed on the following rations: (1) stock ration; (2) ration 800 plus 2% 1:20 liver extract; (3) ration 800. The animals of the group receiving ration 800 grew poorly, averaging 3.5 gm. per week. Four of these animals showed a loss of hair on the sides of their bodies. Since Woolley ('41) reported that inositol is an antialopecia factor for mice, it was thought this factor might be required by the cotton rat.

To study the effect of adding inositol and p-aminobenzoic acid to the synthetic ration, three groups of three rats each were fed the following diets: (1) ration 800 plus 2% 1:20 liver extract; (2) ration 801 (800 plus 100 mg. of inositol and 30 mg. of p-aminobenzoic acid per 100 gm. of ration); (3) basal ration 800. The animals receiving inositol and p-aminobenzoic acid grew considerably better than those on the basal ration but poorer than those which received the liver extract. The growth rates were 8.6, 5.6 and 2.9 gm. per week respectively. The individual effects of these compounds on growth were then studied. Four groups of rats were fed the following rations: (1) ration 800; (2) 800 plus 30 mg. of p-aminobenzoic acid per 100 gm. of ration; (3) 800 plus 100 mg. of inositol per 100 gm. of ration; (4) ration 801. Rats which received inositol alone grew at approximately the same rate as those receiving ration 801 (5.8, and 5.2 gm. per week respectively), whereas those receiving the p-aminobenzoic acid alone grew at a rate of 2.9 gm. per week.

Since the cotton rat could be maintained on a synthetic diet a study of the other vitamin requirements was possible. The thiamine, riboflavin, pantothenic acid, pyridoxine, and nicotinic acid requirements have been studied as well as additional growth promoting substances found in crude materials.

Thiamine

To determine the approximate requirements of thiamine the following experiment was set up: A thiamine-free synthetic ration similar to ration 801 in respect to the other vitamins and supplemented with 2% sulphited 1:20 liver extract

was used for the basal ration. Five groups of rats were given this ration supplemented with 0, 40, 60, 80 and 150 μ g. of thiamine per 100 gm. of ration. All rats on every level except the 150 μ g. level became polyneuritic before the experiment terminated. The rats on the 150 μ g. level grew at a rate comparable to the growth obtained on the optimum synthetic diet, 5.2 gm. per week, but the growth was less than was observed on a synthetic diet supplemented with liver extract. The same convulsive characteristics are observed with polyneuritis in cotton and white rats.

Riboflavin

For a study of the riboflavin requirements a basal diet (801) without riboflavin was supplemented with 0, 25, 50, 80 and 300 μ g. of riboflavin per 100 gm. of ration and was fed to five groups of three rats each. All rats survived the experimental period and grew 1.1, 1.0, 1.7, 3.0 and 6.4 gm. per week respectively. The rats on low riboflavin diets showed no skin symptoms, but those on the suboptimal levels of riboflavin were extremely nervous and excitable, whereas those on the complete ration were more docile. Eighty micrograms of riboflavin per 100 gm. of ration is suboptimal for normal growth of the cotton rat on our synthetic diet.

Pantothenic acid

A synthetic ration which contained 0, 200, 400, 600 and 800 μ g. levels of pantothenic acid per 100 gm. was fed to five groups of cotton rats. In contrast to the reports on the white and piebald rats neither greying of the fur nor adrenal hemorrhage was observed; however animals on the basal ration developed a severe dermatitis. The skin over the entire body became crusted and cracked open, causing large sores. None of these animals survived the 6 weeks experimental period. Rats which received the 200 μ g. level of pantothenic acid grew poorly, whereas those on the higher levels, 400, 600 and 800 grew at approximately the same rate, 5.0, 5.0, and 5.3 gm. per week, respectively, but those which received the 800 μ g.

level appeared to have a better skin and fur coat than those on the lower levels. The growth responses with the same levels of pantothenic acid are similar to those observed with the piebald rat (Henderson et al., '42).

Pyridoxine

Experiments similar to those above were devised to study the pyridoxine requirement of the cotton rat. A synthetic diet free of pyridoxine and containing only 2% corn oil was used as the basal ration. Groups of three rats each were placed on the basal ration supplemented with 0, 30, 60 and 100 μ g. of pyridoxine per 100 gm. of ration. The various levels of pyridoxine gave a better growth curve than any of the other vitamins, being 2.2, 4.0, 5.0 and 6.6 gm. per week for the basal and respective levels. Symptoms other than poor growth in pyridoxine deficient animals were absent. The hair coat was excellent in most cases although the nose and mouth were slightly inflamed on some of the animals on the basal ration. However, there was no evidence of scaly skin or dermatitis.

Nicotinic acid

Cotton rats placed on a synthetic diet free of nicotinic acid for a 6-week experimental period have consistently shown poorer growth than those on a complete synthetic ration containing 2.5 mg. of nicotinic acid per 100 gm. of ration. Several of the rats on the basal ration died before completion of the experimental period. No severe mouth lesions have been noted but in some cases the mouths of cotton rats on the basal ration appeared to be slightly inflamed. Rats on the basal ration grew at an average rate of 2.9 gm. per week. Those on the complete ration grew at an average of 5.7 gm. per week. No further attempts have been made to determine the level of nicotinic acid required by the cotton rat.

Crude materials

Previous experiments indicated that synthetic diets supplemented with 1:20 liver extract produced greater growth re-

TABLE 1
Growth rates of cotton rats on synthetic rations.

NUMBERS OF RATS	GROWTH gm./wk.	NUMBERS OF RATS	GROWTH gm./wk.
<i>Inositol studies</i>			
Steenbock Stock ration.....	15	<i>Pantothenic acid</i>	
Synthetic 801 + 2% 1:20 liver ex- tract.....	9	Synthetic 801 — pantothenic acid (pantothenic acid basal).....	10
Synthetic 800 (no inositol or p- aminobenzoic acid).....	14	Basal + 200 µg. pantothenic acid/ 100 gm.....	3
Synthetic 800 + 30 mg. p-amino- benzoic acid/100 gm.....	3	Basal + 400 µg. pantothenic acid/ 100 gm.....	2
Synthetic 800 + 100 mg. inosi- tol/100 gm.....	6	Basal + 600 µg. pantothenic acid/ 100 gm.....	3
Synthetic 801 (synthetic 800 + 30 mg. p-aminobenzoic acid + 100 mg. inositol/100 gm.....	34	Basal + 800 µg. pantothenic acid/ 100 gm.....	3
<i>Thiamine</i>			
Basal synthetic thiamine ration + 2% sulphited 1:20 liver extract	6	<i>Pyridoxine</i>	
Basal + 40 µg. thiamine/100 gm. ration.....	3	Synthetic 801 — B ₆ (B ₆ basal).....	5
Basal + 60 µg. thiamine/100 gm. ration.....	3	Basal + 30 µg. B ₆ /100 gm.....	3
Basal + 80 µg. thiamine/100 gm. ration.....	3	Basal + 60 µg. B ₆ /100 gm.....	3
Basal + 150 µg. thiamine/100 gm. ration.....	2	Basal + 100 µg. B ₆ /100 gm.....	3
<i>Riboflavin</i>			
Synthetic 801 — riboflavin (ribo- flavin basal).....	9	<i>Nicotinic acid</i>	
Basal + 25 µg. riboflavin/100 gm. Basal + 50 µg. riboflavin/100 gm. Basal + 80 µg. riboflavin/100 gm. Synthetic 801 (300 µg. riboflavin/ 100 gm.).....	3 3 3 3	Synthetic 801 — nicotinic acid.....	14
		Synthetic 801.....	13
<i>Factors in crude products</i>			
Synthetic 801.....	34	Synthetic 801 + 2% 1:20 liver ext.	5.6
Synthetic 801 + 2% 1:20 liver ext.	6	Synthetic 801 + 4% 1:20 liver ext.	8.6
Synthetic 801 + 4% 1:20 liver ext.	9	Synthetic 801 + 6% 1:20 liver ext.	8.2
Synthetic 801 + 2% sol. liver ext....	2	Synthetic 801 + 2% sol. liver ext....	5.2
Synthetic 801 + 4% sol. liver ext....	2	Synthetic 801 + 4% sol. liver ext....	5.7
Synthetic 801 + 5% skim milk powder.....	4	Synthetic 801 + 5% skim milk powder.....	4.1
Synthetic 801 + 1.5 µg. biotin/10 gm.....	3	Synthetic 801 + 1.5 µg. biotin/10 gm.....	4.2
Synthetic 801 + 5% grass juice powder.....	3	Synthetic 801 + 5% grass juice powder.....	4.9
			6.8

sponses than the synthetic diet alone. Three groups of rats were fed ration 801 supplemented with 2, 4, and 6% 1:20 liver extract to determine the level at which optimum growth could be obtained. The rats receiving the 2 and 4% levels grew at approximately the same rate, 8.6 and 8.2 gm. per week respectively. This was considerably better than the growth obtained on the 6% level which was approximately the same as that obtained on the synthetic alone, 5.2 gm. per week. The animals on the 6% level suffered from diarrhea which may account for the poor growth obtained on this level.

Other crude materials were fed to determine the distribution of the growth promoting substances that appear in the 1:20 liver extract. Solubilized liver extract was fed at levels of 2 and 4%. Rats on these rations failed to show any increase in growth over those which received the synthetic ration. The respective weight gains were 5.7 and 4.1 gm. per week. Synthetic diets supplemented with 5% skim milk powder produced even poorer growth than the synthetic ration alone, 4.2 gm. per week. Addition of a biotin concentrate at a level which supplied 15 μ g. per 100 gm. of ration failed to produce any growth response over that obtained on ration 801 alone. On the other hand when the synthetic ration 801 was supplemented with 5% of dried grass juice a slight growth response was noted. Rats on this ration grew at a rate of 6.8 gm. per week. The average weekly gains of all animals studied are shown in table 1.

DISCUSSION

Our results with the breeding and weaning of cotton rats for experimental purposes closely parallel the work reported by Meyer and Marsh ('43) except that our animals were kept on shavings. The absence of feces in the cages indicated that considerable coprophagy takes place. Furthermore, we have not been able to obtain reproduction with animals on our stock ration when they are kept on screens. No difficulties with parasites or infection have been encountered, and no alopecia has been observed in rats on the stock ration. None of our animals are sufficiently tame to handle with ease although

several animals seem tamer than the original stock. However, the maintenance of stock animals and the production of young for experimental purposes can easily be accomplished with proper care.

The B vitamin requirements of the white rat and the cotton rat are similar in many respects, table 2. For optimum growth on the synthetic ration these animals require a minimum of 150 μ g. of thiamine per 100 gm. of ration. Eighty micrograms of riboflavin were not sufficient to maintain the cotton rat, but 300 μ g. per 100 gm. of ration were adequate. Pantothenic acid

TABLE 2
Vitamin B requirements of the cotton rat compared to the white rat.
(μ g. per 100 gm. of synthetic ration)

	COTTON RAT	WHITE RAT
Thiamine	150	80-150
Riboflavin	80 > < 300	100-150
Pyridoxine	100	80-100
Pantothenic acid	800	800
Nicotinic acid	< 2,500	Not required
Inositol	< 100,000	Not known
Choline	< 100,000	< 100,000

must be supplied at a level of 800 μ g. per 100 gm. of ration to obtain optimum growth and appearance. One hundred micrograms of pyridoxine are sufficient. Preliminary work indicates that choline is also required by the cotton rat. The addition of biotin to our synthetic ration did not produce additional growth.

On the other hand, it was necessary to add nicotinic acid and inositol to the synthetic ration to obtain normal growth. The minimum requirements for these substances have not been determined. Levels of 2.5 mg. of nicotinic acid and 100 mg. of inositol per 100 gm. of the ration were used.

A further increase in growth with the addition of 1:20 liver extract indicates the presence of other factors which are necessary for the maximum growth of this animal. These factors which are necessary for maximum growth are not supplied by

solubilized liver which contains the biologically active substance, "folic acid". Skim milk powder also failed to show any activity, although dried grass juice powder seemed to have a limited amount of the active materials.

SUMMARY

Cotton rats require thiamine, riboflavin, pantothenic acid, pyridoxine and choline in approximately the same quantities as the white rat; in addition they require nicotinic acid and inositol. Rats on a completely synthetic diet grow at a rate of only 5.6 gm. per week.

Additional growth promoting substances are present in 1 : 20 liver extract. This is indicated by the increase in growth rate to 8.6 gm. per week when 2% liver extract is added to the ration.

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THE EFFECT OF EXCESSIVE DIETARY SODIUM AND POTASSIUM ON THE CARBOHYDRATE METABOLISM OF NORMAL RATS^{1 2}

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TWO FIGURES

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INTRODUCTION

A number of studies of blood sugar levels have suggested that sodium salts potentiate the action of insulin and that potassium salts have an antagonistic action toward this hormone (McQuarrie, Thompson and Anderson, '36, Adlersberg and Wachstein, '37, Silvette, Britton and Kline, '38 and Orten and Devlin, '40). Many of these reports indicate further that the action of the sodium salts is manifested to a greater degree in diabetic than in normal animals. Administered electrolytes have also been shown to affect the deposition of liver glycogen (Crabtree and Longwell, '36; Silvette, Britton and Kline, '38), a fact which suggests that the blood sugar changes may be secondary to changes in the rates of glycogenesis and glycogenolysis. That a change in the rate of oxidation of glucose may also be a factor is indicated by the reports of Johnston and Maroney ('35), Bruman and Finkelstein, ('36) and McQuarrie, Thompson and Anderson ('36).

¹ From a dissertation presented to the Graduate School of the University of Colorado by Robert C. Lewis, Jr., in partial fulfillment of the requirements for the Ph. D. degree.

² Presented in part before the American Physiological Society, Chicago, Illinois, in April, 1941.

The present investigation was conducted in an attempt to clarify the mode of action of the electrocytes in influencing carbohydrate metabolism in the albino rat. Glucose tolerance, the tolerance to administered insulin, glycogen storage and respiratory quotients were compared in rats which had been maintained on diets differing only in their content of sodium chloride and potassium chloride.

EXPERIMENTAL

Animals and diets

Male rats of the Yale strain which weighed about 200 gm. were maintained on a basal diet to which different salt mixtures were added. The paired feeding technique was used during a preliminary period of 2 weeks. Each group consisted of three animals: one received the unmodified control diet, another the high sodium diet and the third the high potassium diet. The basal diet consisted of starch, 450 gm., casein, 325 gm., unsalted butter, 80 gm., cod liver oil, 20 gm., dried yeast, 80 gm. and powdered agar, 20 gm. per kilogram of diet. The control diet contained 25 gm. per kilogram of a salt mixture which was essentially mixture 351 of Hubbell, Mendel and Wakeman ('37). The high sodium diet contained 20 gm. of a potassium free salt mixture and 90 gm. of extra sodium chloride per kilogram of mixed diet. The high potassium diet contained 20.5 gm. of a sodium free salt mixture and 50 gm. of extra potassium chloride.

Glucose tolerance tests

At the end of the feeding period the groups of three animals were fasted 24 hours and their tolerance was determined to either 0.25 gm. or 0.5 gm. of glucose per 100 gm. of body weight administered by stomach tube. The blood sugar analyses were done by the micro method of Somogyi ('37) on duplicate 0.05 ml. samples of blood from the tail vein.

The range and distribution of the fasting blood sugar levels were approximately the same in each dietary group. The tol-

erance of animals on the three diets to 0.25 gm. of glucose per 100 gm. of body weight showed no consistent differences. When the tolerance was determined following the administration of 0.5 of glucose per 100 gm., the animals on the high sodium diet demonstrated a tolerance which was slightly greater than that of the controls and those on the high potassium diet. Figure 1 shows the mean values for seven animals on each diet. In five of the seven groups the animal

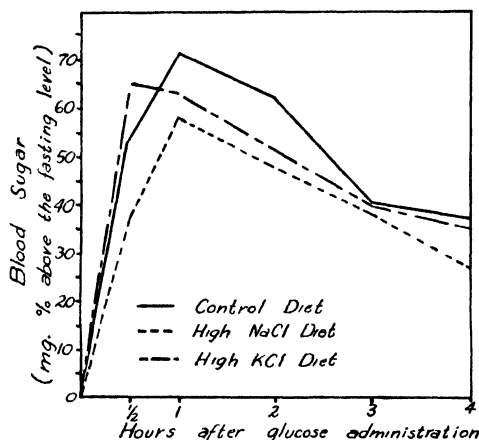


Fig. 1 Glucose tolerance curves after the oral administration of 0.5 gm. of glucose per 100 gm. of body weight. The values show the mean of the determinations on seven animals on each diet.

on the high sodium diet had a lower curve. However, statistical analysis by the *t*-test³ reveals that only the values at the second hour after glucose administration are significantly different ($P < 0.02$). There was no significant difference in the glucose tolerance between the control animals and those fed excessive potassium.

Insulin tolerance tests⁴

The preliminary feeding period and 24-hour fast were the same as in the previous experiment. Each animal then re-

³Significant differences for small groups of animals were determined by "Student's" method (cf. Fisher, '34). By this method significant differences are indicated when $P = 0.05$ or less (Fisher, p. 158).

⁴The insulin used was furnished by Eli Lilly and Co., Indianapolis, Indiana. We gratefully acknowledge their cooperation.

ceived 0.3 to 0.5 units (the same within each group) of regular insulin subcutaneously per 100 gm. of body weight. Figure 2 gives the mean blood sugar values for seven groups of animals. In six of the animals fed the high sodium diet the initial fall of the blood sugar was faster than in the corresponding control. Application of the *t*-test to these values for the first half hour following the administration of insulin shows that $P < 0.05$. Values for the first, second and third hour blood sugars are not significantly different. The return

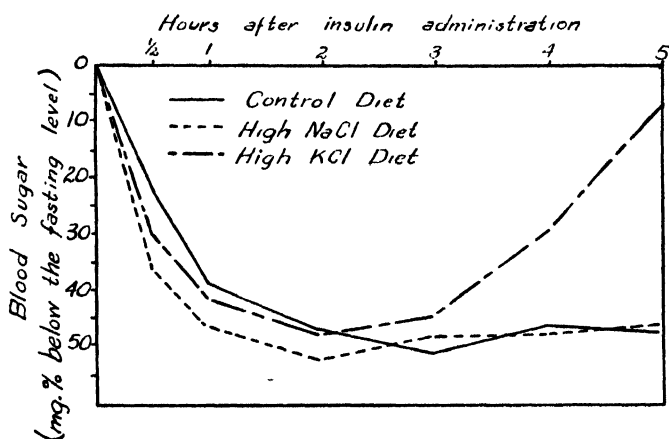


Fig. 2 Blood sugar curves after the administration of insulin. The values show the mean of the determinations on seven animals on each diet.

of the blood sugar toward the normal level at the 4-hour period was consistently faster in the animals which had been fed the high potassium diet than it was in the others. The difference between these animals and the controls is significant ($P < 0.05$). Insulin shock occurred in five of the animals on the high sodium diet, in three of the controls, and in only one of those on the high potassium diet.

Glycogen deposition

Nine groups of animals were maintained through a preliminary feeding period as described above. After a 24-hour fast they were given 0.25 gm. of glucose per 100 gm. of body

weight by stomach tube. Two hours later they were anesthetized with sodium amytal. The livers were removed and frozen immediately in a mixture of solid carbon dioxide and ether, and the gastrocnemius muscles were frozen in situ. These tissues were crushed to a fine powder (cf. Graeser,

TABLE 1
Determinations of glycogen and respiratory quotients.

THE VALUES FOR LIVER AND MUSCLE GLYCOGEN 2 HOURS AFTER THE ADMINISTRATION OF 0.25 GM. OF GLUCOSE PER 100 GM. BODY WEIGHT FOLLOWING A 24-HOUR FAST							
DIET	NUMBER OF ANIMALS	MUSCLE GLYCOGEN GM. PER 100 GM.			LIVER GLYCOGEN GM. PER 100 GM.		
		High	Low	Mean	High	Low	Mean
Control	9	0.57	0.43	0.50	2.76	1.30	2.01
High NaCl	9	0.60	0.45	0.52	2.15	1.55	1.79
High KCl	9	0.61	0.42	0.52	2.47	1.21	1.59

VALUES FOR LIVER GLYCOGEN AFTER A 12-HOUR STANDARD FEEDING PERIOD FOLLOWING A 24-HOUR FAST

DIET	NUMBER OF ANIMALS	LIVER GLYCOGEN GM PER 100 GM		
		High	Low	Mean
Control	10	5.96	2.20	4.41
High NaCl	10	7.41	3.40	5.26

MEAN VALUES FOR RESPIRATORY QUOTIENTS AFTER THE ADMINISTRATION OF 0.25 GM OF GLUCOSE PER 100 GM BODY WEIGHT FOLLOWING A 24-HOUR FAST

DIET	NO OF ANIMALS	RESPIRATORY QUOTIENTS					
		Fasting	Hours after glucose				
			0-1	1½-2½	3-4	4½-5½	7½-8½
Control	8	.732	.806	.857	.816	.766	.727
High NaCl	8	.723	.818	.775	.755	.727	..
High KCl	8	.730	.798	.829	.779	.758	.729

Ginsberg and Friedemann, '34), mixed and sampled in duplicate for analysis by the method of Good, Kramer and Somogyi ('33). The results are summarized in table 1. The amount of glycogen in the muscles was approximately the same in the three dietary groups. The storage of liver glycogen by the animals on the high sodium diet did not differ

significantly from that of the controls. However, the animals which received the high potassium diet consistently stored less glycogen than the controls. The t -test for significance of this difference shows that $P < 0.01$.

A study of the effect of a high sodium diet on liver glycogen storage by a different technique is also summarized in table 1. The glycogen determinations were made following the principles outlined by Guest⁵ ('41). Following the preliminary feeding period the rats were fasted 24 hours. They were then given 5 gm. of the mixed diet and 12 hours were allowed for its consumption. At the end of the 12 hours the liver glycogen was determined in the manner described above. The glycogen storage of animals which had received the high sodium diet was greater than that of the controls in eight of ten pairs. The t -test shows that this difference is significant ($P < 0.05$).

Repetition of the studies of Crabtree and Longwell ('36), in which the liver glycogen was determined after a 24-hour fast, failed to show any significant difference between control animals and those fed excessive sodium. The individual variations in a series of twenty-two pairs were very large, and there was no consistent trend in the animals which had received the experimental diet. The mean values for experimental and control animals were 0.285 gm. and 0.338 gm. per 100 gm. of liver, respectively.

Respiratory quotient studies

Eight groups of animals were studied. At the end of the preliminary feeding period the animals were fasted for 24 hours and the gaseous exchange for a 1-hour period was determined by the method of Haldane (1892). The rats were then given 0.25 gm. of glucose per 100 gm. of body weight by stomach tube and the gaseous exchange was determined at intervals thereafter. Each determination was carried through a 1-hour period, and the recorded R. Q. represents

⁵ Dr. Guest outlined his method to us in 1939, prior to the publication of his results. We are indebted to him for this information.

the total respiratory exchange for that hour. The results of this experiment are recorded in table 1. There was virtually no difference between the fasting R. Q. of the three dietary groups. The change during the first hour after the administration of glucose was of about the same magnitude in all three groups. The R. Q. of the animals which had received high sodium diets then decreased during the second hour, whereas that of the control animals and those on the high potassium diet continued to rise. The R. Q. of the former animals had returned to the fasting level within 5 hours, whereas it was still considerably above that level in animals on the other diets. Application of the *t*-test to these data shows that $P < 0.01$ for the $1\frac{1}{2}$ to $2\frac{1}{2}$ -hour period, $P < 0.05$ for the 3 to 4-hour period and $P < 0.01$ for the $4\frac{1}{2}$ to $5\frac{1}{2}$ -hour period. All of these differences are significant. The differences between the control animals and those on the high potassium diet were not significant.

DISCUSSION

If insulin sensitivity is judged by the initial rate of fall of blood sugar after insulin administration, by the incidence of insulin reactions, and by the rate of return of the blood sugar to normal, it can be concluded that the high sodium diet induced an increase in the sensitivity to insulin and that the diet high in potassium made the animals less sensitive. Glucose tolerance tests, on the other hand, showed only a questionable increase in the ability of the animals fed excessive sodium to utilize glucose, and they failed to show that animals fed excessive potassium had any definite change in their glucose tolerance. Inasmuch as many factors other than insulin are involved in the utilization of ingested glucose, these latter findings do not vitiate the conclusion that sensitivity to insulin was influenced by the dietary regimes employed. The choice of the Yale strain of rats for this study of "normal" animals was perhaps unfortunate in the light of the report by Cole and Harned ('38) which showed that this strain has a diabetic tendency. Their finding was confirmed

by Orten and Devlin ('40), who observed a more marked effect of sodium chloride injection on glucose tolerance in "low tolerance rats" than in "normal rats." It is possible that more significant differences in glucose tolerance might have been observed in our work if a preliminary division into low tolerance and normal animals had been made.

The liver glycogen storage following the ingestion of glucose when the preliminary diet contained excessive sodium chloride was not significantly different from that of controls. However, when a 12-hour standard feeding period was used instead of the single dose of glucose, the high sodium animals stored significantly greater amounts of liver glycogen than did the controls. It should be noted that in the standard feeding period the animals received considerably more potential glucose as starch, protein and fat than did those which received a single dose of glucose. The failure to confirm the results of Crabtree and Longwell ('36) may have been due to the difference in the strain of rats used. These workers used animals from the Denver University colony. Application of the *t*-test to the individual data (unpublished) of their second series of animals shows that the differences were significant ($P < 0.01$).

Rats on a high sodium diet had a lower R. Q. following the ingestion of a measured amount of glucose than did animals on a control diet. This finding may reflect the tendency of the former to deposit more liver glycogen and thus render less glucose available for oxidation. Parallel studies of intestinal absorption and glucose excretion would probably make possible a more exact interpretation. Sayers and Orten ('41) demonstrated that there was more glycosuria when glucose in saline was administered by the intraperitoneal route than when the same amount of glucose was given in distilled water. Our procedure was not comparable to that of Sayers and Orten, but, if electrolyte feeding at this level did increase glycosuria, then our findings may have been partially conditioned by this reaction. The decreased deposition of glycogen in rats which had received a high potassium diet should

have resulted in an increase in the amount of glucose available for oxidation and, therefore, in an increase in the R. Q. when compared to that of control animals. However, the results herein reported do not indicate a significant difference in the R. Q. of the two dietary groups. It is possible that here, too, the excessive electrolyte may have induced an increased glycosuria which influenced the R. Q.

An exact explanation of the results herein reported is not immediately evident. It may be that changes in blood sugar and in the oxidation of glucose are the result of primary changes in glycogen storage, but unequivocal proof of this possibility is lacking.

SUMMARY

Male albino rats of the Yale strain were maintained for a period of 2 weeks on normal diets and on diets high in sodium or potassium. After this period determinations were made of the glucose tolerance, the response to insulin, the deposition of glycogen following the feeding of glucose or a measured amount of mixed diet, and the R. Q. in fasting animals and after the administration of glucose.

Whereas statistical analysis showed a significant difference in the values at only one point in the glucose tolerance curves, the curves of the animals fed excessive sodium tended to be lower than those of the control rats when 0.5 gm. of glucose per 100 gm. of body weight was given.

The animals which received excessive sodium were more sensitive and those which received excessive potassium were less sensitive to administered insulin than were those on a control diet.

When the liver glycogen was determined after a 24-hour fast there were no significant differences between the dietary groups. Following the administration of a measured dose of glucose the liver glycogen of the animals on excessive potassium was significantly lower than that of the control animals. Those which had received excessive sodium did not vary sig-

nificantly from the controls under these conditions. However, when a standard feeding of mixed diet was given, the results showed a significant increase in liver glycogen storage by the animals receiving excessive sodium. The dietary regimes employed had no significant effect upon the muscle glycogen.

The increase of the R. Q. following the administration of glucose was neither as marked nor as prolonged in the animals which had received excessive sodium as it was in those on a control diet. The rats which had received excessive potassium did not vary significantly from the controls.

ADDENDUM

Sayers, Sayers and Orten ('43), have recently published a report showing an increased utilization of glucose by Yale strain rats which were given sodium chloride along with glucose by intraperitoneal administration. Their results show a lower blood sugar when NaCl is given with glucose than with glucose alone. They also observed higher liver glycogen values in their NaCl treated animals. These results are in accord with our findings. They also report higher carcass glycogen in their NaCl treated animals. We did not find a significant difference in muscle glycogen values in our various groups but their findings on the whole carcass probably are more significant inasmuch as the values for one muscle do not represent a true picture of total tissue glycogen changes. These workers conclude that the effect of NaCl is to increase the storage of carbohydrate in the experimental animal, a conclusion which we have also reached by an entirely different technique.

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THE RETENTION OF THE NUTRITIVE QUALITY OF BEEF AND PORK MUSCLE PROTEINS DURING DEHYDRATION, CANNING, ROASTING, AND FRYING¹

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The use of large quantities of meat by the armed forces and for export makes it more necessary than ever to know whether canning, dehydrating, or ordinary cooking have any damaging effect on the nutritional quality of the proteins in the meat. The researches reported on the effect of heat on meat proteins are not sufficiently harmonious to constitute a clear-cut answer to the question.

Some reports (Jarussowa, '29; Scheunert and Bischoff, '30; Scheunert and Venus, '32; Seegers, Schultz and Mattill, '36; Swanson and Nelson, '38) support the view that moist heat, such as is used in boiling and autoclaving, does not significantly lower the nutritional quality of meat proteins. On the other hand, Morgan and Kern ('34), believe that autoclaving or boiling does damage these proteins. As to the influence of dry heat, Mendel and Lewis ('14) reported that low temperature drying (55°C.) of proteins in meat had practically no effect. More severe heating (above 100°C.) causes considerable injury (Seegers and Mattill, '35). Feeding tests have shown that the drying of meat at temperatures below 80°C. gives a product whose protein is equal in nutritive quality to that of cooked meat (Bate-Smith, '42). Pro-

¹ Presented before the Division of Biological Chemistry, Detroit meeting, American Chemical Society, April 12, 1943.

teins in fish meals are apparently damaged by dry heat (Ingvaldsen, '29; Maynard and Tunison, '32; Maynard, Bender, and McCay, '32; Schneider, '32). A lowering of the digestibility appears to be largely responsible for the decreased nutritional quality of dry-heated proteins.

EXPERIMENTAL PROCEDURES

The meats studied

Raw cured pork shoulder. Fresh pork shoulders which had been boned, ground and dry-cured. After grinding the meat was mixed mechanically with the dry curing salts and then vacuumized long enough to remove air. Curing required 24 to 48 hours at 32-34°F.

Canned cured pork shoulder. A portion of the above raw cured pork which was vacuum sealed in 2½- or 6-pound tin cans and kept at 235°F. in a commercial retort until a center temperature of 225°F. was reached. The smaller cans required 3 hours whereas the larger cans required 3½ hours.

Roast pork shoulder. Fresh pork shoulders (butt end removed) which were roasted, two in each oven, in open trays without added water at 325° F. until an internal temperature of 185°F. had been reached, requiring 4½ to 5 hours. After removal of skin, bone, and excess fat, the meat from the shoulders was ground and mixed.

Fried pork shoulder butt. Fresh pork shoulder butts (from the shoulders above) which were sliced ½ inch thick and fried in open skillets. The slices were first quickly browned on each side at relatively high temperatures, then fried at a lower temperature for 15 minutes on each side. After removal of excess fat and bone the meat from the butts was ground and mixed.

Dehydrated pork. Fresh pork shoulders which had been boned and cut into 2-inch cubes, pre-cooked a minimum of 30 minutes after the internal temperature reached 165°F., the broth drained off, the meat ground and dehydrated to 10% moisture content in a Roto-Louvre drier at an air tempera-

ture of about 155°F. Two to 3 hours were required for drying. The broth, after removal of the fat, was concentrated and mixed with the dehydrated meat. The finished product was compressed into tin cans and sealed under vacuum.

Dehydrated beef. Fresh carcass beef which had been boned and cut into 2-inch cubes, pre-cooked a minimum of 30 minutes after the internal temperature reached 165°F., ground and finally dehydrated to 10% moisture content in a Roto-Louvre drier at an air temperature of about 155°F. Dehydration was completed in 2 to 3 hours. Finished product was packed like dehydrated pork.

In all cases the above meats were mixed into the respective diets without additional treatment other than the necessary grinding for proper mixing, and freezing for preservation until used. Dehydrated meats were reconstituted with water before mixing into the diets when comparisons were made with moist meats.

When feeding raw pork, the rats were examined for trichinae at the termination of the experiment to insure freedom from such infestation unless the pork samples had been frozen long enough to kill trichinae before starting the experiment (United States Department of Agriculture, '40).

All meats were analyzed for moisture, protein, and fat as a basis for designing the diets.

Six male weanling albino rats were started in each group of test animals. They were grouped with respect to both size and litter except in experiment 4 where rats (Sprague-Dawley) of unknown litter origin were used. All rats were housed in an air-conditioned room maintained at nearly constant temperature and humidity. Ad libitum feeding was used in all experiments except experiment 2 where paired feeding was employed. Individual weekly weight records were kept for growth periods of 8 to 10 weeks. Individual food consumption records were kept for those animals which were caged separately, but not for those in experiment 3 which were grouped six to a cage. A group food record was kept in this experiment.

The efficiency of protein utilization for growth, as proposed by Osborne et al., ('19), i. e., gain in weight per gram of protein eaten, was used as the criterion of protein quality.

The diets used

Usually all meat proteins were fed at two levels. At the higher levels, usually 17 to 20% of the solids, 10 to 15 % of the total calories were contributed by the protein. This may be considered as a practical level since the data of Berryman and Chatfield ('43) show that protein supplied 12.1% of the total calories in the United States Army diets during a 6-month period. Also Sherman ('41) states that “. . . the results of recent research confirm and strengthen the generally accepted dietetic custom of allowing from 10 to 15% of the total food calories for protein under ordinary conditions.”

The lower levels of protein, 8 to 11% of the solids or 7 to 9% of the total calories, were suboptimal for rat growth and, therefore, were suitable for a more critical evaluation of the proteins.

All diets were analyzed as a further check on their composition and the protein contents given are values calculated to the dry weight basis from the analytical data. In order that the protein contents given might represent the meat protein content, corrections were applied for the non-meat nitrogen contributed by yeast, liver concentrate powder, and corn starch when present.

With the exception of experiment 1-b, the meats were added to each diet by replacement of a weight of carbohydrate equal to that of the meat solids (dry solids were calculated from analytical data for each meat). Other ingredients are given on an air-dry basis except in experiment 4 where all ingredients are on a dry-weight basis.

The diets used in the respective experiments had the following composition:

Experiment 1-a. Each of the two meats, raw cured pork and canned cured pork, was used in amounts to provide 10,

20, and 29% of pork protein in the diets. The fat contents of the two comparable diets at each protein level were made equal by adding lard in the amount required to the diet having the lower fat content. It was necessary to omit the butter from the basal ration to make the diets containing 29% of pork proteins because of the high fat content of these diets.

The balance of the diets contained salt mixture, 5 (that of Phillips and Hart '35, modified by the addition of 0.9 gm. $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ per kilogram); butter, 6; dried brewer's yeast, 5; fortified sardine oil (3000 A, 400 D), 1; liver concentrate powder (Wilson's 1:20), 1; and dextrose to make 100%.

The B-complex content of the diets was supplemented by giving each rat a daily aqueous supplement of the following synthetic vitamins: thiamine, riboflavin, and pyridoxine, 20 $\mu\text{g.}$ each; calcium pantothenate, 100 $\mu\text{g.}$; niacin, 1 mg.; and choline chloride, 50 mg.

Experiment 1-b. These two diets were prepared with a protein distribution as follows: Raw cured pork or canned cured pork, 7.5% of the diet; wheat, 4.8; oats, 3.3, and corn, 2.1. The complete diets contained pork (wet basis), 25%; wheat, 30; oats, 20; corn, 14; salt mixture, 5; butter, 5; and fortified sardine oil, 1.

The B-complex content of these diets was not supplemented.

Experiment 2. Raw cured pork and canned cured pork were each used at levels to provide 9.7 and 18.8% protein in the diets. Lard was added to give a protein to fat ratio of 1:2 and salt mixture and calcium carbonate were adjusted to give a calculated total calcium and phosphorus content of 1.00 and 0.67% of the solids respectively. The balance of each diet consisted of dried brewer's yeast, 2; fortified sardine oil, 1; liver concentrate powder (Wilson's 1:20), 0.5; and corn starch to make 100%.

The B-complex content of each diet was supplemented as in experiment 1-a except that the choline chloride was reduced to 15 mg. and the supplement was administered in dextrin pills.

Experiment 3. The diets used here were the same as those in experiment 1-a except that unreconstituted dehydrated beef and dehydrated pork were used as sources of protein. Both beef and pork were fed at a protein level of 20% and also at levels of 10 and 11% respectively.

Tallow was added to the beef diets to give a protein to fat ratio of 1 : 2. The B-complex content of these diets was not supplemented.

Experiment 4. Meats were used to provide protein levels as follows: Canned cured pork, 8.9 and 18.3%, roast fresh pork, 9.2 and 17.7%, fried fresh pork, 9.1 and 17.4%, reconstituted dehydrated pork, 8.3 and 17.1%, reconstituted dehydrated beef, 8.3 and 18.1%. Fat was adjusted by the addition of lard to the pork diets and tallow to the beef diets to give a protein to fat ratio of 1 : 2. Salt mixture and calcium carbonate were proportioned to give calcium and phosphorus values of 1.00 and 0.67% of the solids respectively. Fortified sardine oil was included at a 1% level and corn starch was used to bring the total solids to 100%.

The natural B-complex content of the diets was supplemented by giving daily to each rat one 50 mg. protein-free yeast extract tablet (purchased from Harris Laboratories, Tuckahoe, New York). The adequacy of B-complex intake was confirmed by the daily addition of 0.8 gm. of liver concentrate powder to the food of each rat beginning with the eighth week and continuing until the end of the experiment (end of tenth week). The growth rate was not changed by the addition.

RESULTS

The results obtained are summarized in table 1. The average gains and protein intakes for the entire growth periods of 8 to 10 weeks have been tabulated. Whenever differences were observed between comparable groups, they usually became evident about the second week and increased progressively to about the eighth week. The largest differences were observed at about the eighth to tenth week.

TABLE 1
Nutritive value of meat proteins.

EXP. NO.	PROTEIN SOURCE	PROTEIN LEVEL	NUMBER OF RATS	GROWTH PERIOD	WEIGHT GAIN AVG.	PROTEIN INTAKE AVG.	GRAMS OF GAIN PER GRAM OF PROTEIN	
							Avg.	Range
		%		weeks	gm.	gm.		
1-a	Raw cured pork	10	5	8	180	66	2.73	2.39-2.91
	Canned cured pork		5		159	60	2.65	2.40-2.79
	Raw cured pork	20	5	8	210	112	1.87	1.76-1.95
	Canned cured pork		5		204	106	1.92	1.79-2.14
	Raw cured pork	20	6	8	213	148	1.44	1.35-1.50
	Canned cured pork		6		207	147	1.41	1.31-1.51
1-b	Raw cured pork + cereals	17.7	6	8	207	128	1.62	1.41-1.78
	Canned cured pork + cereals		6		181	117	1.55	1.35-1.69
	Raw cured pork	9.7	6	10	135	62	2.18	1.95-2.42
2	Canned cured pork		6		124	62	2.00	1.59-2.22
	Raw cured pork	18.8	6	8	191	100	1.91	1.81-2.02
	Canned cured pork		6		194	99	1.96	1.71-2.29
3	Dehydrated pork	11	6	9	174	72	2.42	
	Dehydrated beef	10	6		160	61	2.62	Group
	Dehydrated pork	20	6		237	127	1.98	caging
	Dehydrated beef		6	9	226	120	1.97	
	Canned cured pork	8.9	5		126	55	2.29	2.20-2.40
4	Roast fresh pork	9.2	5	10	149	65	2.29	2.20-2.33
	Fried fresh pork	9.1	6		175	68	2.57	2.51-2.66
	Dehydrated pork	8.3	6		155	56	2.77	2.62-2.86
	Dehydrated beef	8.3	6		114	50	2.28	2.05-2.40
	Canned cured pork	18.3	6		256	144	1.78	1.64-1.90
	Roast fresh pork	17.7	6	10	262	139	1.88	1.75-2.09
	Fried fresh pork	17.4	6		282	146	1.93	1.76-1.99
	Dehydrated pork	17.1	5		259	139	1.86	1.73-2.03
	Dehydrated beef	18.1	6		262	149	1.76	1.67-1.80

In experiment 1-a, no difference was found in the efficiency of utilization by rats of the proteins of raw cured pork and canned cured pork at either the 20 or 29% level of protein. At the 10% level of protein, a slightly higher efficiency of utilization was observed for the raw cured pork proteins, although inspection of the respective ranges of individual values indicates the difference may not be a real one.

In experiment 1-b, a cereal mixture fed with raw cured pork also showed a slightly higher efficiency of protein utilization than the same cereal mixture with canned cured pork. Although the range of individual values indicates less variation within these groups than at the 10% level above, the difference is very small if real.

In both experiments 1-a and 1-b, the values obtained for protein intake are apparent rather than absolute since they were calculated from the measured food intake with no correction for evaporation losses. The diets were of comparable moisture content and the protein efficiency values obtained should, therefore, be comparable, although they may run somewhat lower than values which would have been obtained if the absolute protein intake had been determined.

In experiment 2 the paired feeding technique was employed to eliminate the variations in protein intake observed in the previous ad libitum experiments. The data in tables 1 and 2 show no difference in the efficiency of protein utilization at the higher protein level. A statistical analysis (see Snedecor, '40) indicates that the difference between the protein efficiencies of raw cured and canned cured pork at the sub-optimal level is slightly significant (probability of one in twenty that it could be due to chance). It is, therefore, probable that the canning process lowers the nutritive quality of the proteins to a small extent.

The uneaten food was assayed for moisture content in this experiment to permit measurement of the absolute food intake from which the absolute protein intake was then calculated.

In experiment 3, a comparison of the efficiencies of utilization of dehydrated beef and pork proteins showed a slightly

higher value for dehydrated beef at the suboptimal level. It is difficult to determine whether or not this may be a real difference, but it is definitely not a great one. There is no difference at the higher level of protein intake.

The diets were air-dry and the values obtained are, therefore, absolute. The values are quite similar to those obtained by Hoagland and Snider ('26) for ox and hog muscle using a similar method, i. e., growth of male rats for a 60-day period at 10 and 15% protein levels. Their values for ox muscle

TABLE 2

Paired feeding data for rats fed raw cured and canned cured pork shoulder—Experiment 2.

GROWTH PERIOD	PORK PROTEIN LEVEL	PAIR NO.	DIET	FINAL WEIGHT	INITIAL WEIGHT	WEIGHT GAIN	PORK PROTEIN INTAKE	GRAMS OF GAIN PER GRAM OF PROTEIN
<i>Weeks</i>	<i>%</i>			<i>(gms.)</i>	<i>(gms.)</i>	<i>(gms.)</i>	<i>(gms.)</i>	
10	9.7	1	Raw	191	62	129	62.4	2.07
			Canned	186	63	123	61.6	2.00
		2	Raw	200	69	131	67.1	1.95
			Canned	181	75	106	66.5	1.59
		3	Raw	216	59	157	64.9	2.42
			Canned	200	58	142	64.9	2.19
		4	Raw	178	56	122	54.6	2.23
			Canned	178	56	122	55.0	2.22
		5	Raw	211	57	154	70.3	2.19
			Canned	201	55	146	68.1	2.14
		6	Raw	165	48	117	52.5	2.23
			Canned	154	49	105	54.7	1.92
8	18.8	1	Raw	216	61	155	78.1	1.98
			Canned	205	62	143	83.2	1.72
		2	Raw	250	68	182	100.5	1.81
			Canned	275	70	205	100.8	2.03
		3	Raw	246	61	185	99.2	1.86
			Canned	224	62	162	94.6	1.71
		4	Raw	262	57	205	109.8	1.87
			Canned	306	59	247	108.0	2.29
		5	Raw	263	49	214	105.9	2.02
			Canned	245	51	194	101.8	1.91
		6	Raw	276	72	204	107.4	1.90
			Canned	277	65	212	108.3	1.96

at 10 and 15% protein levels were 2.55 and 1.7 respectively; for hog muscle the corresponding values were 2.46 and 1.87 respectively. Since their samples were air-dried below 60°C. for about 24 hours, probably a less severe process as regards proteins than dehydration, this comparison with their values may afford presumptive evidence that the dehydration process does not damage the proteins of pork and beef to any great extent.

The results of a comparison of the protein quality of canned cured pork shoulder, roast fresh pork shoulder, fried fresh pork shoulder, dehydrated pork and dehydrated beef in experiment 4 placed dehydrated pork first, fried fresh pork shoulder second, and the remaining three of equal quality when compared at a suboptimal level of intake. The differences at this level were not very large. At a higher and more practical level of protein intake no differences in protein quality were observed between the five meats.

DISCUSSION

Since the evidence obtained shows some probability of a slight damage to the proteins of cured pork shoulder by the commercial canning procedure described, this may be an indication that a more severe processing schedule might damage the pork proteins to a greater extent than this processing schedule.

These investigations have not determined whether the schedules used in canning other meats, these schedules often being more severe than for cured pork, are damaging to the respective proteins, and also whether the nutritional quality of the muscle proteins of other species is changed by heat treatment.

CONCLUSIONS

It is indicated by the efficiency of protein utilization for rat growth that: (1) The nutritive quality of the proteins of cured pork shoulder may be slightly lowered by a commercial canning process. (2) The proteins of dehydrated pork muscle and fried fresh pork shoulder are indicated to be slightly

superior in nutritive quality to those of canned cured pork shoulder, roast fresh pork shoulder, and dehydrated beef muscle.

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THE AVAILABILITY OF THE CALCIUM AND PHOSPHORUS OF DEFLUORINATED ROCK PHOSPHATE FOR THE RAT

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TWO FIGURES

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The critical shortage of bone meal and calcium phosphate has stimulated a search for other mineral supplements which would be a satisfactory source of phosphorus for animal feeding. Raw rock phosphates and superphosphates can not safely be used in animal feeds because of their high fluorine content. However, methods have been developed by which rock phosphates and superphosphates can be defluorinated. The fluorine content can be reduced to less than 0.10%, which is below the toxic level for a mineral supplement in the feeding of any farm animal according to Mitchell ('43). Because relatively little is known about the availability for bone growth of the calcium and phosphorus of defluorinated rock phosphates this problem has been studied.

Three defluorinated rock phosphates have been considered. The calcium, phosphorus and fluorine content of these products and of bone meal and secondary calcium phosphate are shown in table 1.

Products nos. 1 and 2 were defluorinated by a superphosphate procedure, that is, the rock phosphate was treated with sulfuric acid to form superphosphate and then the excess acid and the fluorine were driven off by heat treatment. Product no. 3 was defluorinated by a fusion procedure. In this process

silica is added to the rock phosphate and the mixture is fused to expel the fluorine.

Cannon ('38) concluded that the calcium of product no. 2 was well utilized by growing chicks. Fraser et al. ('43) in experiments with a fused rock phosphate such as product no. 3 found the phosphorus to be well utilized by rats.

TABLE 1
Analysis of the products used in the experiments.

MINERAL SUPPLEMENT	CALCIUM	PHOSPHORUS	FLUORINE
	%	%	%
Product no. 1	25.71	13.72	0.04
Product no. 2	28.22	12.49	0.05
Product no. 3	30.80	12.67	0.04
Steamed bone meal	33.58	15.00	0.04
Calcium phosphate	29.45	22.79	0.00

EXPERIMENTAL

In a preliminary experiment product no. 1 was compared with bone meal at a level which furnished 0.26% of phosphorus in the diet. The basal diet was essentially the same as that used by Schneider and Steenbock ('39), and contained 0.046% phosphorus and 0.016% calcium. Eight pairs of female litter-mate rats weighing approximately 36 gm. at 24 days of age were selected and pair-fed. After 35 days the rats were sacrificed. Both femurs were removed and as rapidly as possible freed from the adhering flesh. They were first weighed in water and then weighed suspended in air. Specific gravity determinations were made according to the following formula:

$$\text{Specific gravity} = \frac{\text{Weight of bones in air}}{\text{Weight of bones in air} - \text{weight of bones in water}}$$

The excised femurs were then allowed to dry for 3 or 4 days at room temperature and x-ray pictures were made. They were then dried at 100°C., extracted with anhydrous ethyl ether and ashed at 600°C. This procedure of analyzing the femurs was used in subsequent trials. The average results of these determinations as well as the growth data clearly

showed that bone meal was superior to product no. 1 for bone formation in the rat. The growth of all the rats was rather poor, however. This was thought to be due to inadequate protein in the basal ration.

The experiment was repeated using a basal ration modified to include some blood fibrin. The basal diet (table 2) contained 0.060% of phosphorus and 0.027% of calcium. In this experiment, bone meal was compared with two levels of product no. 1 and one level of product no. 3. The mineral supplements were added to the basal feed to give diets containing 0.26% of

TABLE 2
Composition of basal diet.

	%
Sucrose	51.68
Egg white (cooked)	13.00
Fibrin (alcohol extracted)	5.00
Starch (cooked)	20.00
Vitab ¹	4.00
Wesson salt ²	1.32
Wesson oil	5.00
Vitamins A and D concentrate ³

¹ A rice bran concentrate from National Oil Products Company.

² Ca and P-free modification of Wesson's ('32) salt mixture.

³ Four drops per rat per week, supplying 680 I.U. of D, and 5,000 I.U. of A. Product of the White Laboratories, Inc., Newark, N. J.

phosphorus and 0.46% of calcium. A higher level of product no. 1 was also added to give a diet containing 0.42% of phosphorus and 0.75% of calcium.

Ten trios of female litter-mate rats were used to test product no. 1 and seven pairs to study product no. 3. The growth data for the 35-day test and the average results of the analyses of the femurs are shown in table 3.

From these data it is clear that product no. 1 was inferior to bone meal. The rats fed bone meal made more growth and developed larger and more dense bones than those fed product no. 1. Even when product no. 1 was fed at an appreciably higher level it did not produce as good results as did bone meal. In sharp contrast product no. 3 permitted equal growth

and fully as large and dense femurs as when bone meal was fed.

In a third experiment it was decided to compare three defluorinated rock phosphates at four levels of intake. Secondary calcium phosphate was used as a standard. An experiment of factorial design was selected because it establishes a curve of response for each supplement and permits the simultaneous comparison of the effects of any level of one product with any level of another. The basal diet was the same as that used in the second experiment (table 2).

TABLE 3

A comparison of mineral products no. 1 and no. 3 with bone meal

SUPPLEMENT	NUMBER OF RATS	FOOD EATEN	GAIN IN WEIGHT	FEMUR ANALYSES			
				Specific gravity	Weight of femur ash	Ash content	Ash per cubic millimeter
		gm.	gm.		mg.	%	mg.
Product no. 1	10	262	60.8	1.26	106.5	55.0	0.34
Bone meal	10	262	73.0	1.44	200.4	64.1	0.55
Product no. 1 ¹	10	262	57.8	1.32	128.4	58.1	0.40
Product no. 3	7	277	75.4	1.44	191.1	63.2	0.55
Bone meal	7	277	77.6	1.47	202.8	64.0	0.57

¹ This diet contained 0.42% of phosphorus and 0.75% of calcium.

The phosphorus supplements to be tested were added in varying amounts to the basal mixture to give diets which contained 0.15, 0.25, 0.50 and 1.00% of phosphorus. The calcium content of the diets was adjusted with calcium carbonate so that all diets contained a Ca: P ratio of about 2: 1.

One female and one male rat at 24 days of age were allotted at random to each of the diets. The average initial weight of the females was 34.3 ± 2.6 gm. and for the males 33.4 ± 2.1 gm. All animals were fed equal caloric amounts of the diets, adjustments being made if the rats began to leave food.

During the 35-day trial the rats on the lowest level of mineral supplement ate 227 gm. of their diet, while those on the higher level of mineral supplement received slightly more. The rats on the highest level of product no. 1 ate about 90%

of their food. The general appearance of all the rats was normal.

The average weight gains of the rats on the different supplements at the various levels of calcium and phosphorus intake are shown in figure 1. Although the gains were somewhat irregular, due to animal variation, it can be noted that the weight increase of the rats on calcium phosphate product no. 2, and product no. 3 were essentially the same. The curve of gain in weight for the rats on product no. 1 shows a downward trend, however, as the intake of this mineral was increased. This indicates that some factor was progressively

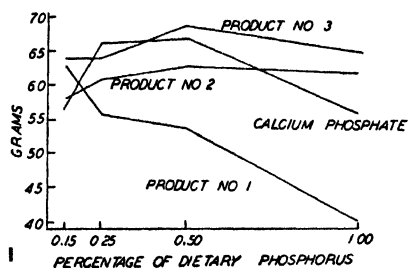


Fig. 1 The gain in weight of rats fed the mineral supplements at increasing levels.

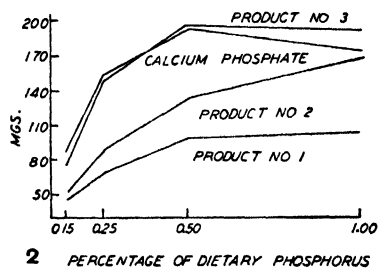


Fig. 2 The total femur ash of rats fed the mineral supplements at increasing levels.

inhibiting growth as the level was raised. The depression of the growth of the rats due to product 1 was statistically significant.

The average total ash of the femurs for the male and females on the various diets at each level is presented graphically in figure 2. This criterion shows the greatest differences of all the measures used in this study. One observes that the femur ash for the rats receiving product no. 3 and calcium phosphate are almost identical, indicating the same utilization of these products at the various levels. It is observed that 0.25% phosphorus from product no. 2 gave about the same femur ash as 0.15% from calcium phosphate and that 0.50% of

phosphorus equaled 0.25% from calcium phosphate. Thus it appears that product no. 2 is only about one-half as available for bone formation as calcium phosphate. The femur ash of the rats receiving product no. 1 was definitely below all the others. At 0.50% of phosphorus product no. 1 approximately equaled 0.15% from calcium phosphate, thus indicating that product no. 1 was only about one-third as available for bone formation as the calcium phosphate.

The average per cent ash of the dry fat-free femurs is shown in table 4. Here one observes that product no. 3 supported about the same bone growth as calcium phosphate. The differences in the per cent ash between these products

TABLE 4
The percentage ash in the dry fat-free femurs.

SUPPLEMENT	LEVEL OF PHOSPHORUS				MEAN OF DIETS
	0.15	0.25	0.50	1.00	
	%	%	%	%	%
Calcium phosphate	53.55	61.84	65.25	64.42	61.27
Product no. 1	40.73	49.02	54.36	60.38	51.12
Product no. 2	44.28	51.39	60.86	63.74	55.07
Product no. 3	49.57	60.48	65.39	65.52	60.24
Mean of levels	47.03	55.69	61.46	63.52	56.92

are not significant. The femurs of the rats which received product no. 2 had a significantly lower per cent ash than did the femurs of the rats receiving the calcium phosphate. The rats receiving product no. 1 had the lowest ash percentage in their femurs.

Determinations of the specific gravity and the ash per unit of volume showed the same results as the other measures. The data are omitted in order to conserve space.

X-ray pictures were taken of the excised femurs in all of the experiments and in some cases full-body x-ray pictures were also made. This was done in order to obtain a further check upon the extent of bone calcification. The x-ray findings agreed with and supported the growth and bone data that are presented.

DISCUSSION

Both products no. 1 and no. 2 exhibited low availability for bone formation in the rat. The reason why these products were poorly utilized is not known. Both of these products were defluorinated superphosphates, and the presence of relatively large amounts of calcium metaphosphate may account for their low availability. MacIntire ('37) states that the phosphorus of raw rock phosphates exists as the ortho form. However, it is possible that calcium metaphosphates are produced during the superphosphate defluorination process.

Mitchell ('43) reports that the phosphorus of calcium metaphosphate prepared from rock phosphate is poorly utilized by the rat. Fraser et al. ('43) reports that the phosphorus of both calcium metaphosphate, c.p. and calcium metaphosphate produced from rock phosphate were poorly utilized by the rat. These workers did not state whether the calcium metaphosphate was amorphous or crystalline. MacIntire reports that in the production of calcium metaphosphate from rock phosphate one can get two types of metaphosphate depending upon the treatment. If the product is air-cooled a crystalline-like product of very low solubility results; whereas, if the hot product is quenched, a glassy-type, amorphous calcium metaphosphate which is much more soluble is obtained. It is possible that these two types of calcium metaphosphate differ in their availability to the animal organism, but the question needs to be studied.

It is suggested that the low availability of the defluorinated superphosphates, studied in these experiments, may be due to the presence of the lesser available calcium metaphosphate.

SUMMARY

A basal diet low in both calcium and phosphorus has been employed to study the availability of three defluorinated rock phosphates for bone formation in the rat. The rock phosphates have been studied at levels of from approximately 0.7 to 7.0% of the diet. These levels gave diets containing from 0.15%

phosphorus and 0.24% calcium to 1.00% phosphorus and about 2.0% calcium.

Product no. 1, a defluorinated superphosphate, was significantly less efficient for bone formation than calcium phosphate or bone meal. Product no. 1 inhibited the growth of rats, and this became more apparent as the level was raised.

Product no. 2, a defluorinated superphosphate, was less available for bone formation than calcium phosphate at low levels. However, when it was supplemented at approximately twice the level of calcium phosphate, equal bone formation resulted.

Product no. 3, a fused rock phosphate, was slightly less efficient than calcium phosphate at very low levels. When the diet was supplemented with this fused, defluorinated product to contain 0.50% phosphorus or more, however, product no. 3 was equally as satisfactory as calcium phosphate for bone formation.

It is suggested that the low availability of the defluorinated superphosphates, products 1 and 2, may be due to the presence of relatively large amounts of the poorly utilized calcium metaphosphate.

While these results may not apply to farm animals they suggest the importance of giving attention to procedures used in manufacturing defluorinated rock phosphates and of testing these products with farm animals.

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THE COPPER METABOLISM AND REQUIREMENT OF YOUNG WOMEN¹

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ONE FIGURE

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The meagerness of the data available on the copper requirement of human beings is understandable when one remembers that only 15 years ago was copper added to the list of elements known to be essential to the animal organism.

One of the first suggestions as to the amount of copper needed for the maintenance of man was made by Tompsett ('34) as a result of metabolism studies conducted on some of his patients. One of these subjects on the very low copper intake of 0.21 mg. daily excreted 0.63 mg. per day. Since 0.63 mg. was needed to replace the amount lost, Tompsett suggested this amount as a minimum daily requirement. Chow and Adolph ('35) studied the copper metabolism of four normal adults for periods of from 1 to 3 days and found that equilibrium was reached on a daily intake of about 2.0 mg. of copper. Ohlson and Daum ('35) present figures for the copper intake and output of three young women for periods of 5 to 15 days in which the daily intakes ranged from 0.96 mg. to 1.15 mg. and in every case the excretion exceeded the intake by amounts from 0.08 to 0.40 mg. Orr ('35) made a dietary survey of the poorer classes in Aberdeen and used the presence or ab-

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sence of anemia as the criterion of adequacy of the copper content of the diet. In the non-anemic families the average daily intake of copper was 2.88 mg. and he suggested that the daily adult requirement was, therefore, no greater than 2.88 mg.

The data for the present report are from two sources: Ninety-five 1-week metabolism studies of sixty-five healthy young women who were living on their customary self-chosen diets, to be designated as series I; and a metabolism study of four healthy young women on an adequate constant diet for a continuous period of 75 to 140 days, to be designated as series II. The details of the set-up and procedure for the two studies respectively are reported by Leverton and Marsh ('42) and by Leverton and Roberts ('37). In both series composites were made of the food eaten by each subject and all excreta were collected for analysis.

Two colorimetric methods were used for the determination of copper, the one of Fischer and Leopoldi ('34) employing diphenylthiocarbazone and Coulson's ('37) modification of the Haddock-Evers method employing diethyldithiocarbamate. Comparison of results obtained by using both methods for the same materials showed excellent agreement. A photo-electric photometer was used for measuring the intensity or concentration of the color complex.

RESULTS

Because much of the value of the results lies in the number of subjects and, therefore, the cross section of metabolic activity that is represented for young women of this age, it is the plan to present average figures of all subjects and groups of subjects rather than figures of individual subjects. Throughout, the results of series I and series II will be presented together but will not be combined because series I includes many subjects for a short period whereas, series II includes a few subjects for a long period. The copper metabolism data for series I, sixty-five subjects on their customary self-chosen diet, and for series II, four subjects on an adequate constant diet, are given in table 1. The data have been sorted in two

TABLE 1

Copper metabolism data for all subjects and for different groups of subjects arranged according to the level of intake and according to the performance of the subjects.

DESCRIPTION	NO. STUDIES	NO. DIFFERENT SUBJECTS	AVERAGE DAILY				
			Intake	Coef. of variation	Excretion	Coef. of variation	Retention
			mg.	%	mg.	%	mg.
Series I Self-chosen diets	95	65	2.65±.0517	28.1	1.80±.0369	29.5	0.85±.0490
Series II Constant diet	76	4	2.37±.0354	19.3	2.14±.0293	17.7	0.23±.0268

Data sorted according to size of intake

Range of daily intake							
Series I							
1.00-1.99	16	15	1.60±.0528	19.6	1.57±.0653	24.7	0.03±.0560
2.00-2.99	51	38	2.48±.0272	11.6	1.71±.0455	28.3	0.77±.0388
3.00-3.99	23	20	3.42±.0377	7.9	2.10±.0778	26.4	1.32±.0739
4.00-4.99	5	5	4.21±.0428	33.6	2.11±.1655	26.0	2.10±.1369
Series II							
1.00-1.99	18	3	1.82±.0215	7.4	1.89±.0324	10.8	-.07±.0338
2.00-2.99	51	4	2.44±.0285	12.4	2.20±.0365	17.6	0.24±.0387
3.00-3.99	7	2	3.21±.0653	8.1	2.37±.0733	14.7	0.84±.1116

Data sorted according to performance of subjects

Performance ¹							
Series I							
Negative	8	8	1.85±.1121	23.7	2.35±.1250	20.9	-.50±.0630
Equilibrium	9	9	2.22±.2167	40.9	2.19±.2155	41.1	0.03±.0149
Positive	78	56	2.78±.0517	24.2	1.70±.0311	23.8	1.08±.0410
Series II							
Negative	18	4	2.04±.0495	15.3	2.39±.0675	17.8	-.35±.0268
Equilibrium	10	4	2.03±.0575	13.3	2.01±.0535	12.5	0.02±.0095
Positive	48	4	2.56±.0131	16.6	2.08±.0332	16.4	0.48±.0314

¹ A storage or loss no greater than 5% of the intake was not considered significant and therefore was counted as equilibrium.

ways: first, according to the level of intake in order to compare the excretion and retention of copper at progressively greater intakes, and second, according to performance, that is whether the intake equaled, exceeded, or was exceeded by the excretion, thus indicating that the subject was in equilibrium, positive, or negative balance.

The average daily copper intake for all the subjects in series I was 2.65 mg., the excretion 1.80 mg. with the subsequent retention of 0.85 mg. In series II the intake was slightly less, 2.37 mg., the excretion slightly greater, 2.14 mg., and the retention, therefore, only 0.23 mg. The majority of the intakes in both series was between 2.00 mg. and 2.99 mg. of copper per person per day and on this intake some of the metal was retained. In series I, 82% of the studies showed the subjects to be storing copper and in series II, 63% of the studies showed copper storage. There were only eight cases in series I in which the amount of copper that was excreted in the urine and feces exceeded the intake, nine cases in which the excretion equaled the intake, and in the remaining seventy-eight cases the copper intake exceeded the excretion and there was an average daily retention of 1.08 mg. In series II there were forty-eight cases of copper retention, ten cases of equilibrium, and eighteen cases of negative copper balance of which fifteen were for the same subject; however, this subject was in copper equilibrium when the entire study was considered.

When the present data are sorted according to the size of the intake it may be noted that the excretion did not increase at the same rate as the intake and, therefore, there was an increased retention at each successively higher level of intake. The relation of the intake to the retention of copper is shown graphically in figure 1. For series I the $r = .6903 \pm .036$, and for series II $r = .5810 \pm .051$.

Of particular importance, if the results are to be used as a basis for determining requirement, is the relation between increases in intake and subsequent increases in retention. Expression of this relationship is somewhat involved, for it is not the percentage of the intake that is retained but the per-

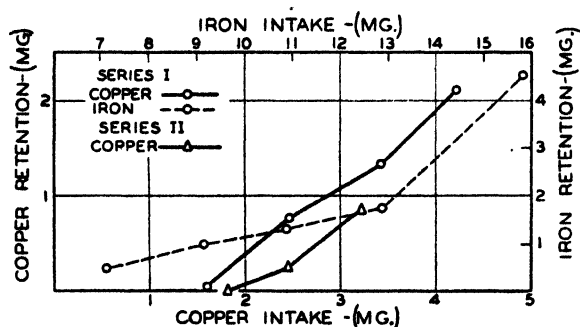


Fig. 1 The relationship between the level of intake and the retention of copper and iron for each series and of copper for series II.

centage of the increase in intake which is retained that may give the pertinent information. In table 2 are shown the increases in the average amounts of copper ingested and retained by groups of subjects at one level of intake over the average performance of the subjects in the group at the next lower level. The most significant column is the one which shows the percentage of each additional intake that was retained. It appears that a large proportion of each increase in copper intake was retained rather than unabsorbed or

TABLE 2

Relationship of increases in level of intake to increases in level of retention for copper and iron.

NUTRIENT	INCREASE IN AVERAGE DAILY INTAKE			INCREASE IN AVERAGE DAILY RETENTION			INCREASE RETAINED
	From mg.	To mg	Actual mg	From mg	To mg	Actual mg	
Copper for series I	1.60	2.48	0.88	0.03	0.77	0.74	84.1
	2.48	3.42	0.94	0.77	1.32	0.55	58.5
	3.42	4.21	0.79	1.32	2.10	0.78	98.7
Copper for series II	1.82	2.44	0.62	-0.07	0.24	0.31	50.0
	2.44	3.21	0.77	0.24	0.84	0.60	77.9
Iron for series I	7.16	9.18	2.02	0.41	0.97	0.56	27.7
	9.18	10.87	1.69	0.97	1.34	0.37	21.9
	10.87	12.91	2.04	1.34	1.76	0.42	20.6
	12.91	15.85	2.94	1.76	4.55	2.79	94.9

excreted and that an increase in the intake to over 4.0 mg. still did not result in a diminished rate of absorption. The iron data for the subjects in series I which show the same high percentage absorption suggest the intestinal tract may treat the two metals in much the same manner.

The copper content of sixteen menses for the four subjects in series II was determined, and unlike the menstrual losses of iron by these same subjects (Leverton and Roberts, '36) varied greatly from one period to another. The average loss per period for each of the four subjects was 0.32 mg., 0.48 mg., 0.65 mg., and 0.74 mg. with an average of 0.55 mg. for all the subjects. The copper content of the menses determined by analysis was twelve times greater than when it was calculated from the volume of blood lost (calculated in turn from the iron content) and the average figure for copper content of blood. The subject with the largest copper losses in the menses had the lowest hemoglobin (12.3 gm. per 100 ml.) and the subject with the highest hemoglobin (13.5 gm. per 100 ml.) had the lowest copper losses. A third subject had an increase from 12.0 to 14.0 gm. hemoglobin over a 90-day period and during that time the copper content of the menses fell from 0.74 mg. in the first period to 0.49 mg. in the second, to 0.22 mg. in the third menstrual period. The fourth subject followed no discernible pattern.

No relationship could be found between the copper intake and retention and the hemoglobin values in the subjects of series I, and studies were not made during menstruation of the subjects.

The correlation between the intake of copper and iron, and between the excretion of copper and iron are as follows:

	SERIES I	SERIES II
Copper intake and iron intake	0.318 \pm .0625	0.746 \pm .0343
Copper excretion and iron excretion	0.380 \pm .0595	0.517 \pm .0567

There was no evidence that the level of copper intake influenced iron storage or vice versa. In series I the daily copper intake of the subjects who stored iron was 2.72 mg. as compared with 2.47 mg. by those who did not store iron, whereas

the daily iron intake of the subjects who retained copper was 10.40 mg., and 10.36 mg. by those who did not retain copper. It was not practicable to compare the intake of dietary essentials other than copper, for the group which was storing copper with the group which was not storing copper, because in the latter group there were too few subjects.

DISCUSSION

When results of a study of this kind are used in the determination of human requirement, major interest is focused upon the relation between the intake and the retention, whether positive or negative, of the nutrient under consideration. There are, however, limitations to the interpretation of even the most carefully conducted metabolism studies. Retention has to be calculated from the determination of the intake and of the excretion. Since the intake can be controlled the reliability of figures for retention is dependent upon how accurately the excretion reflects normal customary metabolic activity and how accurately the excretion is measured. There is considerable error inherent in the method of measuring fecal excretion, namely, that the carmine which is given to mark the stools is subject to the vagaries of intestinal action and motility, after which the analytical results depend upon the accurate separation of the marked from unmarked portion of the stools. For this reason small differences in results secured from studies of only a few days' duration must be viewed with extreme conservatism.

The figures for the retention of copper at different levels of intake and the upward trend of the curves shown in figure 1 suggest that the body's inability to excrete iron by way of the gut may also apply to copper. As the intake became progressively greater there was no flattening of the curves to denote a decrease in the proportion of the intake that was retained. Such a decrease could indicate that the current body needs had been met, that the rate of storage had approached its maximum and, consequently, that the unaccommodated copper was being excreted. Instead, the curve continues upward and at the

highest level of intake, much above the average for the majority, 50% of the intake was retained. There is, moreover, no hint that the curve will flatten at any level of copper intake attainable from ordinary foods.

Further evidence that the intestinal tract may handle copper and iron alike is suggested by the results which are plotted in figure 1 and illustrate the similarity between the intake and retention relationships for copper and iron for the same subjects. The actual amount of iron, as well as the percentage of the increase in the intake that was retained (table 2), is greater at each succeeding higher level of intake between 9.18 and 15.85 mg. of iron daily.

If the intestinal tract does not excrete copper, the occurrence of negative balances must have been apparent rather than real, and a result of such factors as: (1) too short a period of study, (2) unusual mixing of the carmine marker with the intestinal contents, (3) difficulty in separating the carmine-marked from the unmarked portions of the feces, (4) an atypical state of the subject, (5) an abnormal permeability of the individual's intestinal tract to the excretion of copper. Although reference to table 1 shows that the intakes of the subjects in negative balance in both series I and series II were smaller and the excretions larger than for the subjects in equilibrium or for those retaining copper, other information regarding them is also pertinent. The eighteen cases of negative balance in series II become insignificant when it is explained that fifteen of these occurred in the same subject and when the entire time of the study was considered she was in copper equilibrium and the other subjects were in positive balance. In series I there were eight cases of negative balance and these subjects had an average of 8.7 stools per subject per week, as compared with the 8.5 for those in equilibrium and the 8.0 for those who retained copper. The likelihood that negative copper balances are atypical permits discussion of some of the implications of the theory that copper is not normally excreted by the body.

If it is true that the intestinal tract permits only one-way passage of copper, and that in the direction of absorption, the

customary metabolism study technique for determining the requirement of a nutrient is inadequate when applied to copper. After this was found to be true of iron it was helpful to study how the body managed on very small amounts of iron (Leverton, '41), but this has yet to be done with copper.

With no indication of excretion of copper through the intestine the loss of copper from the body may be limited to urinary excretion and hemorrhage. This would suggest that the metal is used over and over again as is the case with iron, and, consequently, the requirement would be exceedingly small. The daily retention of 0.04 mg. would replace the urinary loss, which approximates 0.02 mg., and leave 0.02 mg. toward replacement of copper lost in the menses. The level of intake that would insure this theoretical amount of retention can be approximated from table 1. On self-chosen diets, many of which were found by Leverton and Marsh ('42) to be inadequate in respect to calcium, phosphorus, and nitrogen, the average daily intake of copper by all subjects was 2.65 mg. and this amount permitted an average daily copper retention of 0.85 mg.

In the sixteen cases with the lowest copper intake, 1.60 mg., there was a retention of 0.03 mg. and in fifty-one cases with copper intakes between 2.0 and 3.0 mg. (average 2.48 mg.) the daily retention was 0.77 mg.

Also reference to table 2 shows that 84% of the increased intake between the intakes of 1.60 mg. and 2.48 was retained. Considering these lines of evidence 2 mg. of copper would probably be an ample daily allowance for women of this age and certainly an allowance of 2.5 mg. of copper would be above question.

The chances are very good that self-chosen diets of even mediocre quality will contain 2.0–2.5 mg. copper because in the ninety-five studies of these subjects on self-chosen diets there were:

44 cases in which the daily calcium intake was less than 0.8 gm.

87 cases in which the daily phosphorus intake was less than 1.35 gm.

46 cases in which the daily iron intake was less than 10 mg.

78 cases in which the daily protein intake was less than 67 gm.

SUMMARY AND CONCLUSIONS

Data on the copper metabolism of young women are reported from two sources: Ninety-five 1-week studies of sixty-five young women on self-chosen diets, and a long-time study of four young women on an adequate constant diet.

The average daily intake by the subjects on the self-chosen diets was 2.65 mg. of copper and their average daily retention was 0.85 mg. For the subjects on the constant diet the average daily intake and retention of copper were 2.14 mg. and 0.23 mg. respectively.

As the copper intakes increased a large proportion of each increase was retained.

The copper content of sixteen menses for four subjects varied greatly from one period to another.

Considerable evidence is advanced in support of the theory that the body does not excrete copper and that the intestinal tract may handle copper as it does iron.

A daily allowance of 2.0–2.5 mg. of copper is suggested for young women together with evidence that this amount can be obtained from diets of otherwise mediocre nutritive value.

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FOLIC ACID, BIOTIN AND PANTOTHENIC ACID DEFICIENCY AND THE LIVER STORAGE OF VARIOUS VITAMINS IN RATS FED SUCCINYLSULFATHIAZOLE IN HIGHLY PURIFIED RATIONS

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The concentration in the tissues of various vitamins of the B complex has been employed by many investigators as an index of the adequacy of an animal's supply of these dietary essentials. Of the various tissues, changes in the vitamin content of the liver have, in general, shown the best correlation with variations in the vitamin intake and the nutritional status of the animal. In this communication data are presented on the storage of folic acid, biotin, and pantothenic acid in the liver of the rat, as influenced by the incorporation of succinyl-sulfathiazole in highly purified diets adequate in the well-recognized members of the vitamin B complex.

It has been shown (Welch, Mattis and Latven, '42; Welch and Wright, '43) that the inclusion of succinylsulfathiazole in amounts up to 10% of the ration has no demonstrable deleterious effect on rats when the remainder of the diet is composed of relatively crude ingredients (stock ration¹). The inclusion of 1 or 2% of succinylsulfathiazole in rations composed of purified ingredients and containing thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, inositol, p-aminobenzoic acid, and choline in amounts believed to be

¹ The Purina brand was employed as a stock ration in these studies.

adequate, brings about a retardation in the rate of growth of rats. On such rations growth finally ceases and death occurs after a few weeks. Accompanying the effect on growth manifestations of dietary inadequacy such as alopecia, spectacled eyes, ophthalmitis, porphyrin-caked whiskers and achromotrichia may occur. A combination of crystalline biotin and folic acid concentrate ² will effectively prevent or cure the condition. Reasons have been presented elsewhere (Black, Overman, Elvehjem and Link, '42; Martin, '42; Nielsen and Elvehjem, '42; Welch and Wright, '43; Wright and Welch, '43a) for relating the effect of succinylsulfathiazole to its effect on the synthetic activity of intestinal bacteria.

PROCEDURE

Young male black or piebald rats of approximately 50 gm. weight were caged individually over wide mesh screening and were fed ad libitum on the various rations outlined in table 1. After the animals had subsisted on these rations for varying lengths of time, representative animals were killed by decapitation and their livers assayed for several members of the vitamin B complex by microbiological methods. With some of the animals liver autolysates prepared by the method described by Wright et al. ('41) were used. In the remainder of the series the livers were subjected to enzymatic digestion with takadiastase at their natural pH ³ (Cheldelin et al., '42). In our experience autolysis yields approximately 90% (range 89-93%) of the pantothenic acid present in normal liver, based on the amount liberated after enzyme digestion with takadiastase. However, autolysis yields only 41% (range

² The substance termed "folic acid" is probably not a single entity. The evidence for the probable existence of several substances which can be utilized for growth by *L. casei* ϵ or by *Streptococcus lactis* R, but which appear to be of significantly different potency, has been discussed by Stokstad ('43).

³ Two gm. of liver fragmented with a spatula, suspended in 20 ml. water, takadiastase (equivalent to 2% of the liver weight) added, and digestion carried out for 18-24 hrs. at 37°C. under benzene. This, of several procedures, has given the highest values for folic acid in liver without the use of xanthopterin (Wright and Welch, '43b, 43c).

38-43%) of the folic acid obtainable from normal liver by takadiastase digestion. The biotin data were obtained from assays performed on samples of liver which had been digested by autoclaving at 15 pounds pressure for 1 hour with 6N H₂SO₄ and then neutralized with NaOH.

TABLE 1
Composition of diets employed in feeding experiments.

	s-5	s-6	s-6A	s-7	s-8
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Casein (Labco)	18.0	18.0	18.0	18.0	18.0
Fat (Primex)	...	10.0	10.0	10.0	10.0
Corn oil	2.0	2.0	2.0	2.0	2.0
Sucrose	72.4	59.9	59.9	59.9	59.9
Salts ¹	4.0	4.0	4.0	4.0	4.0
Cellu flour	1.5	4.0	4.0	4.0	4.0
A, D, and E concentrate ²	.. ⁴	.. ⁴	.. ⁴	0.08	0.08
Choline chloride	0.1	0.1	0.1	0.1	0.1
Succinylsulfathiazole ³	2.0	2.0	2.0	2.0	2.0
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
Thiamine hydrochloride	3.0	.. ⁵	.. ⁶	0.2	0.2
Riboflavin	3.0	.. ⁵	.. ⁶	0.4	0.4
Pyridoxine hydrochloride	3.0	.. ⁵	.. ⁶	0.2	0.2
Nicotinic acid	2.5	.. ⁵	.. ⁶	4.0	4.0
Calcium pantothenate	2.0	.. ⁵	..	4.4	...
p-Aminobenzoic acid ⁶	4.0	4.0
Inositol ⁶	8.0	8.0
2-methyl-1, 4-naphthohydroquinone diacetate	1.0	1.0

¹ Osborne and Mendel ('13) or Hubbell, Mendel and Wakeman ('37).

² Compounded as follows: fish liver concentrate containing 450,000 U.S.P. units of vitamin A and 90,000 U.S.P. units of vitamin D per gram, 7 gm.; α -tocopherol, 2 gm.; corn oil, 41 gm.

³ In rations containing no succinylsulfathiazole this compound was replaced by an equivalent amount of sucrose.

⁴ Animals in these groups were given 2 drops per week of percomorph oil fortified with α -tocopherol (1 mg. per 25 mg. oil).

⁵ Animals in this group were given a daily subcutaneous injection of 1 ml. of a solution containing (per ml.) thiamine hydrochloride, 10 μ g.; pyridoxine hydrochloride, 10 μ g.; riboflavin, 20 μ g.; nicotinic acid, 200 μ g.; calcium pantothenate, 220 μ g.

⁶ Animals in this group were given a daily subcutaneous injection of 1 ml. of a solution containing (per ml.) thiamine hydrochloride, 10 μ g.; pyridoxine hydrochloride, 10 μ g.; riboflavin, 20 μ g.; nicotinic acid, 200 μ g.; p-aminobenzoic acid, 200 μ g.; inositol, 400 μ g.

In general one microbiological assay for each factor was performed on all the tissues of a given experiment. While there may be slight differences between the results of consecutive assays, all values within a single experiment are strictly comparable.

Riboflavin was determined by the method of Snell and Strong ('39). The determinations of pantothenic acid were carried out by either the method of Pennington, Snell and Williams ('40) or that of Landy and Dicken ('42). The methods of the latter workers were employed in the determinations of folic acid, biotin, and nicotinic acid. Titration of the lactic acid produced after a growth period of approximately 72 hours was employed as a measure of the response of *Lactobacillus casei* to the factor in question. Samples of crystalline biotin from two sources served as reference standards for biotin determinations. A folic acid concentrate⁴ served as the folic acid standard. This was assayed against a sample of liver extract (Wilson's fraction B) and the results have been calculated in terms of micrograms of "potency 40,000 units," as described by Cheldelin et al. ('42).

RESULTS

Wright et al. ('41) and Mitchell and Isbell ('42) have presented data on the B vitamin content of normal rat tissues. We have found that the inclusion of succinylsulfathiazole in highly purified rations does not cause a significant deviation from the normal values for the concentrations of either riboflavin or nicotinic acid in the liver. The level of riboflavin in the livers of several series of sulfonamide-fed rats averaged 25 $\mu\text{g.}$ per gram (range 18–27 $\mu\text{g.}$ per gram). Nicotinic acid levels in several groups of animals, likewise receiving succinylsulfathiazole in highly purified rations, averaged 155 $\mu\text{g.}$ per gram (range 119–189 $\mu\text{g.}$ per gram). Since these observations indicate that no gross change in the composition of the liver occurred during the period of sulfonamide administration, they support the hypothesis that the storage of specific

⁴ Kindly furnished by Dr. E. L. R. Stokstad.

B-vitamins in the liver may be used as a criterion for the estimation of the status of an animal with reference to the nutritive essentials measured.

Liver from animals fed purified rations adequate for normal growth contained only a fraction of the folic acid found in the hepatic tissue of animals subsisting on the stock ration. The inclusion of succinylsulfathiazole in the same purified rations (which rendered them inadequate for normal growth) resulted in only a slight further diminution in the amount of folic acid found in the liver. Animals which were fed rations adequate except that pantothenic acid was omitted, had a slightly higher concentration of folic acid in the liver than did those which consumed entirely adequate synthetic rations (in which neither succinylsulfathiazole nor folic acid were present).

The biotin content of the liver from animals maintained on the stock ration was found to be about 1.5 μ g. of biotin per gram of fresh tissue. When the animals were reared on synthetic rations without added biotin, but adequate in the other well-recognized constituents of the B complex, the biotin content of the liver was considerably depressed, although evidence of a biotin deficiency was either absent or appeared only in a mild form after the animals had subsisted on such rations for several months. The inclusion of 2% succinylsulfathiazole in the ration caused a further depression in the amount of biotin found in the liver and signs of biotin deficiency were frequently evident (Nielsen and Elvehjem, '41). Neumann, Krider and Day ('43) have also studied the biotin deficiency which develops in rats fed a purified diet containing succinylsulfathiazole and p-aminobenzoic acid. Our data suggest that for signs of biotin deficiency to make their appearance in the rat, the biotin content must be reduced to approximately 0.35 μ g. per gram of fresh liver. Two micrograms of biotin per day, administered subcutaneously, were found sufficient to maintain a concentration of biotin, in the liver of rats fed 2% succinylsulfathiazole, equal to that of animals on the stock ration.

The level of pantothenic acid in the liver was uninfluenced by the addition of succinylsulfathiazole to the extent of 10% in the stock ration. However, when succinylsulfathiazole (2%) was added to highly purified rations containing hitherto adequate amounts of pantothenic acid, low concentrations of pantothenic acid in the liver were invariably found. In such cases the content of pantothenic acid in the liver was consistently found to be comparable to the low levels encountered in induced pantothenic acid deficiency, produced by a ration (S-8) containing neither pantothenic acid nor succinylsulfathiazole. Changes seen in pantothenic acid deficiency (Unna, '40; Unna and Richards, '42) such as marked achromotrichia and porphyrin-caked whiskers, were noted when rats were maintained on purified rations to which succinylsulfathiazole was added (2%). Similar observations with regard to achromotrichia have also been described by Martin ('42), who employed sulfaguanidine. Evidence for pantothenic acid deficiency in rats receiving purified rations, low in protein and containing sulfapyridine, has recently been presented by West, Jefferson and Rivera ('43). These workers found that the oral administration of 1 mg. of calcium pantothenate daily caused more or less complete abolition of the signs of pantothenic acid deficiency within a period of 2 to 4 weeks. In our experiments in which the B-vitamins were given subcutaneously (diets S-6, table 1), neither the subcutaneous administration of calcium pantothenate (0.22 mg. daily), nor a marked increase in the amount of the vitamin in the diet (11 mg. per 100 gm.) raised the depressed pantothenic acid content of the hepatic tissue of rats fed succinylsulfathiazole in purified diets (table 2).

Our analyses of the pantothenic acid content of the livers of rats maintained on rations adequate for normal growth, except with respect to pantothenic acid, indicate that evidence of pantothenic acid deficiency in the rat may be anticipated when the concentration in the liver falls below about 50 μ g. per gram.

Pantothenic acid, biotin and folic acid in the liver of rats on various diets.

EXPERIMENT NO.	SUCCINYL-SULFA-THIAZOLE	TYPE OF RATION	DURATION OF EXPERIMENT	PANTOTHENIC ACID	BIOTIN	FOLIC ACID
%			weeks	$\mu\text{g./gm.}$	$\mu\text{g./gm.}$	$\mu\text{g. "potency 40,000"/gm.}$
1	..	S-5 Purified ration	4	145(110-201) (4) ¹	2.0(1.5-2.5) (4) ¹	1.0(0.6-1.7) (4) ¹
1	2	S-5 Purified ration	4	58(46-66) (4)	1.3(1.2-1.5) (4)	0.84(0.56-0.96) (4)
2	2	S-6 Purified ration; B-vitamins subcutaneously	6	41(39-53) (5)	0.89(0.72-1.1) (5)	..
2	2	S-6 + 2 $\mu\text{g.}$ biotin per day subcutaneously	6	..	1.2(1.2-1.3) (3)	..
2	2	S-6 + 16 $\mu\text{g.}$ biotin per day subcutaneously	6	..	1.6(1.4-1.8) (3)	..
2	2	S-6A + 220 $\mu\text{g.}$ Ca. pantothenate per day subcutaneously	6	45(42-50) (3)
2	2	S-6A + 11 mg. % Ca. pantothenate in ration	6	46(44-48) (3)
3	..	S-7 Purified ration	6	81(61-120) (5)	0.81(0.70-1.1) (5)	1.1(0.76-1.6) (5)
3	2	S-7 Purified ration	6	39(32-48) (5)	0.57(0.41-0.73) (5)	0.48(0.17-0.56) (5)
3	..	Stock ration	..	78(62-104) (5)	1.5(1.3-1.7) (5)	13(10-16) (5)
4	..	Stock ration	6	73(67-79) (4)	1.4(1.2-1.7) (5)	..
4	10	Stock ration	6	78(73-88) (4)	1.3(1.0-1.7) (5)	..
5	..	Stock ration	..	89(88-91) (4)	..	20(14-26) (8)
6	..	S-7 Purified ration	8	96(93-99) (2)	0.41(0.40-0.42) (2)	2.6(2.6-2.6) (2)
6	2	S-7 Purified ration	8	44(35-49) (3)	0.29(0.20-0.40) (3)	1.6(1.5-1.6) (3)
6	..	S-8 Purified ration, no pantothenic acid	8	38(33-47) (3)	0.62(0.58-0.65) (3)	3.6(3.5-3.7) (3)
7	..	S-7 Purified ration	12	83(82-85) (2)	0.41(0.38-0.44) (2)	2.5(2.2-2.7) (2)
7	2	S-7 Purified ration	12	40(33-46) (3)	0.25(0.18-0.32) (3)	1.6(1.0-1.9) (3)
7	..	S-8 Purified ration, no pantothenic acid	12	46(44-49) (3)	0.79(0.75-0.84) (3)	4.6(2.4-7.0) (3)

¹ Accompanying the average values presented are (1) the range in values obtained, and (2) the number of animals studied. Biotin extraction was done by acid hydrolysis; pantothenic acid and folic acid by enzymatic digestion, except in experiments 1, 2 and 4 where antoly-sis was employed. All data are based on the weight of fresh liver employed.

TABLE 3

Liver analyses and changes in weight of rats¹ on a purified diet containing succinylsulfathiazole (2%) given various supplements by stomach tube.

DAILY SUPPLEMENT	NO. OF ANIMALS	DURATION OF EXPERIMENT	CHANGES IN WEIGHT	PANTOTHENIC ACID	BIOTIN	FOLIC ACID
		weeks	gm.	CONTENT OF LIVER	CONTENT OF LIVER	CONTENT OF LIVER
None	3	0	0	$\mu\text{g./gm.}$	$\mu\text{g./gm.}$	$\mu\text{g. "potency 40,000 units"/gm.}$
None	3	3	0	40(33-46)	0.25(0.10-0.32)	1.5(1.0-1.9)
Crystalline biotin, 5 $\mu\text{g.}$	3	3	-8	48(47-49)	0.26(0.19-0.31)	1.2(0.72-1.6)
Crystalline biotin, 5 $\mu\text{g.}$ and folic acid concentrate, 20 mg.	3	3	+16	57(55-61)	0.76(0.49-0.97)	1.2(0.96-1.5)
	3	3	+46	81(80-82)	0.89(0.81-0.94)	4.2(2.6-6.1)

¹ These animals had received for a period of 12 weeks the same purified diet (S-7) containing succinylsulfathiazole (2%).

² The concentrate of grass employed contained approximately 800,000 Snell-Peterson units of folic acid per gram. Biotin extraction was done by acid hydrolysis; pantothenic acid and folic acid were determined after takadiastase digestion of tissue samples.

Curative experiments demonstrate that concentrates of folic acid, administered with crystalline biotin, are effective in reestablishing excellent growth in rats which had previously reached a plateau in their growth curve, following the ingestion for several weeks of purified rations containing succinylsulfathiazole. The results obtained from one such experiment are presented in table 3. The folic acid concentrate employed was prepared from a grass juice powder⁵ by a procedure similar to that described by Hutchings, Bohonos and Peterson ('41). Accompanying the resumption of growth and marked improvement in the general condition of the animals, which followed daily oral administration for 3 weeks of a concentrate of folic acid and crystalline biotin, there was a marked deposition in the liver of pantothenic acid, as well as of folic acid and biotin. The amount of folic acid and biotin found in the liver was intermediate between those amounts encountered in rats on synthetic rations adequate for growth, and those found in rats on stock rations. In addition, the simultaneous oral administration of folic acid concentrates and crystalline biotin to the deficient rats, increased the pantothenic acid content of the liver to the same level as that found in rats on the stock ration.

DISCUSSION

Data obtained from the assay of the hepatic tissue of rats receiving synthetic rations containing succinylsulfathiazole parallel the results of growth experiments, and the appearance of signs of deficiency, in that they show such animals to be deficient in both folic acid and biotin. In addition, a failure in the utilization of pantothenic acid has been demonstrated. Supplements of folic acid and biotin permit rats receiving succinylsulfathiazole to grow essentially normally for a period of several weeks (Nielsen and Elvehjem, '42; Martin, '42; Welch and Wright, '43) and folic acid concentrates, in the presence of crystalline biotin, not only cure the achromotrichia encountered in rats receiving sulfanilylguanidine (Martin,

⁵ Supplied through the kindness of Dr. W. R. Graham of the Cerophyl Laboratories.

'42), but also cause a restoration of the utilization of pantothenic acid.

An explanation of this effect of folic acid concentrates and biotin is not yet possible. It is conceivable, however, that folic acid and biotin are involved in enzyme systems to which pantothenic acid is related. Pilgrim, Axelrod and Elvehjem ('42) found that liver slices from rats deficient in either pantothenic acid or biotin showed a decreased rate of pyruvate oxidation. It was concluded that the two factors are associated in metabolism.

Data presented in table 2 indicate that no impairment of pantothenic acid metabolism occurs when high levels of succinylsulfathiazole are included in a ration composed of natural crude materials. When a synthetic diet is fed, which contains succinylsulfathiazole, and an amount of pantothenic acid similar to that found in the stock ration, adequate hepatic storage of pantothenic acid fails, unless folic acid and biotin are given.

The question may be asked whether a certain type of bacterial activity in the intestine is essential for the proper utilization of "uncombined" pantothenic acid. Much information is available to indicate that pantothenic acid, as it occurs in animal and plant tissue (Cheldelin et al. '42), blood (Wright, '42), and yeast cells (Teague and Williams, '42), is largely in a combined or complex state. It is conceivable that folic acid and biotin are required, when succinylsulfathiazole is included in a highly purified ration, to maintain an intestinal flora which in some manner conjugates free pantothenic acid to an active complex or in some other fashion facilitates the utilization of this factor by the rat. It might be pointed out that the inclusion of zinc chloride in synthetic rations has been reported (Gross, Harválik and Runne, '41) to produce signs in experimental animals closely similar to those encountered in pantothenic acid deficiency. Although the mechanism by which the effect is produced has not been explained, it is possible that the action of zinc chloride on the animal results from an influence on the intestinal flora. Such an effect might

be either on pantothenic acid or on the production of folic acid or biotin.

Molitor ('42) states that in the acute stage of induced pantothenic acid deficiency in the dog, the intravenous injection of pantothenic acid results in prompt recovery from the previously critical condition. Accordingly, on the basis of the evidence available at present, it is quite possible that folic acid and biotin produce their effects on pantothenic acid and other systems within the tissues of the animal and not through reactions occurring within the intestinal tract. A comparison of the minimal effective doses administered orally with those given parenterally may yield information bearing on the sites of action of these compounds.

However the effects of these factors are produced, it is clear that when folic acid concentrates and biotin are not administered to rats fed succinylsulfathiazole in highly purified rations, there develops decisive evidence of a failure properly to use pantothenic acid. In addition to a growth defect, black rats turn gray and present other characteristics of pantothenic acid deficiency not primarily because folic acid is a chromotrichial factor per se but because pantothenic acid metabolism is impaired when inadequate amounts of folic acid and biotin are available (Wright and Welch, '43a).

SUMMARY

A study of the storage of riboflavin, pantothenic acid, nicotinic acid, folic acid, and biotin in the liver was made in rats receiving various types of rations. A highly purified diet, adequate in those members of the vitamin B complex required for the production of excellent growth in rats, caused a marked reduction in the hepatic stores of folic acid and biotin compared with the amounts of these factors found in the liver of animals maintained in stock rations. The hepatic storage of these factors was further reduced by the incorporation of succinylsulfathiazole in such synthetic rations. The storage of riboflavin and nicotinic acid was not demonstrably influenced, a finding which indicates that no gross change in the liver

occurred during sulfonamide administration. Despite the presence in the diet of a previously adequate amount of pantothenic acid, the inclusion of succinylsulfathiazole caused a reduction in the pantothenic acid content of the liver to a level as low as that produced by a diet devoid of pantothenic acid. Increasing the dietary intake of pantothenic acid or giving the vitamin parenterally did not cause a renewal of growth, failed to modify the signs of pantothenic acid deficiency, and left unaffected the severely reduced amount of pantothenic acid in the liver. Administration of crystalline biotin and a concentrate of folic acid caused a prompt restoration of growth, recovery from the signs of pantothenic acid deficiency, and a restoration of the pantothenic acid content of the liver to normal. It is suggested that folic acid (or a constituent of the folic acid concentrate employed) and biotin are essential for the maintenance of growth and of the general health of rats given purified rations containing succinylsulfathiazole. At least a portion of the effect of these factors is attributed to their playing an essential role in the utilization of pantothenic acid.

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SECONDARY ANEMIA DUE TO PROLONGED AND EXCLUSIVE MILK FEEDING AMONG SHOSHONE INDIAN INFANTS

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ONE FIGURE

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It has been found in a previous study that certain dietary deficiencies exist among Indians (Pijoan, Elkin and Eslinger, '43). Since numerous vicious circles of disease occur in many Indian children, it is one of the purposes of this communication to present data dealing with the first deficiency which makes its general appearance in Shoshone Indian infants. This deficiency is dependent on a continued and exclusive milk intake of from 1 to 2 years duration which results in an inadequate iron assimilation for hemoglobin formation. The other purpose of this communication is to present a modification of Elvehjem's method (Farrar, '35) of assaying iron in milk.

Recent studies by MacKay ('41) and by Merritt and co-workers ('34) indicate that a considerable part of the iron which disappears from the circulation of an infant during the physiological decrease of hemoglobin is retained for a variable period of time in body stores, but that within a period of a few months a supply of iron is needed for hemoglobin formation. Milk, on the other hand, is significantly low in iron. However, most infants have a sufficient iron reserve for the first few months which compensates for inadequate iron intake. After this period of compensation has transpired, the

relatively long continued feeding of an exclusive milk diet will produce an anemia primarily due to deficiency of iron (Elvehjem and coworkers, '33; Josephs, '34).

The subjects of this study were a group of healthy Shoshone Indian mothers who were admitted to the Western Shoshone Hospital, Owyhee, Nevada, for parturition and who were kept in the hospital for a period of investigation. They were then allowed to return to their dwellings and the studies were continued in some cases in the field. It must be said that in general the feeding of infants on the part of the mothers is from the breast until such time as the child can partake of the regular diet consumed by adults. There appears to be little supplementary feeding, if any, of vegetable purees, eggs or meat.

METHODS

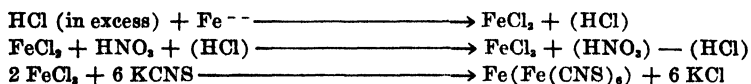
Hemoglobin determinations were carried out on oxalated venous blood by use of the Evelyn and Cipriani method ('37), where the hemoglobin content of 15.6 gm. was equivalent to an oxygen capacity of 20.9 volumes, and represented an empirical hemoglobin value of 100%. In order to rule out primary blood disease, stained blood smears from each subject were studied.

Breast milk was obtained by the use of a breast pump. The milk was dehydrated and preserved for future assays. Elvehjem's method (Farrar, '35) was modified as follows: (a) elimination of iso-amyl alcohol and of potassium permanganate and (b) a change in volume of the other reagents. The elimination of iso-amyl alcohol is a reasonable one in view of the fact that no concentration of chromogen is required when sensitive color measurement apparatus is used; there appears to be no need in such a system of an oxidizing agent such as potassium permanganate. The modification that we suggest is relatively simple.

The reagents should be as iron-free as possible. If traces appear, the blank correction obviates a source of error. The first step is to obtain a calibration constant in which standards containing known amounts of iron are compared to a blank

containing the reagents. The blank is prepared as follows. To a 50-ml. volumetric flask add the following reagents: 5.0 ml. of 20% HCl, 1 ml. of 20% potassium sulfocyanate, 3 drops of conc. HNO₃, and make up to 50 ml. with distilled water. Ten milliliters of this solution is placed in an Evelyn apparatus (Evelyn, '36) and the galvanometer string is set at 100 mm.

An initial stock solution containing a known amount of iron is prepared and subsequent determinations are made on samples from this solution. Five-tenths gram of pure iron wire is dissolved in 20% HCl (sufficient to effect solution) and 1 ml. conc. HNO₃. The solution is evaporated to dryness and the residue is dissolved in 20% HCl, then diluted with water to 1000 ml. Each milliliter then contains 0.05 mg. Fe. Sample portions are then diluted to contain in each milliliter 0.00175 mg., 0.0020 mg., and 0.0015 mg. Each of these subsequent standards (1 ml.) is transferred to 50 ml. volumetric flasks and treated by the reagents used in the blank with the exception that 4 ml. of 20% HCl are used instead of 5 ml. An orange red color develops immediately and is stable for at least 2 hours. This chromogen is due to the formation of a complex salt as follows:



There appears to be sufficient proof (Schlesinger and Van Valkenburg, '31) that the final salt is Fe(Fe(CNS)₆) rather than K₃Fe(CNS)₆ or KFe(CNS)₄.

Since the final chromogen has maximum optical densities in the neighborhood of 429 mμ, a Corning glass filter with transmission limits of 380 mμ to 460 mμ is used. The transmittance of the standard sample solution and that of the blank becomes a ratio. Since the galvanometer is adjusted to 100 with the blank, and the galvanometer reading is proportional to the amount of light transmitted, the following relationship exists: $T = \frac{G}{100}$ where T is the transmittance and G the galvanometer reading. Applying the laws of Lambert and Beer and where C equals the concentration of chromogen (or mg. of substance

responsible for the formation of the chromogen) and K_1 a calibration constant whose value depends on the nature of the chromogen and the characteristics of the filter (Malloy and Evelyn, '37), the following equation is used:

$$C = \frac{1}{K} \times \log_{10} \frac{1}{T} = \frac{1}{K} \times \log \frac{100}{G} = \frac{2 - \log G}{K_1}$$

Thus by determining the deflection caused in the galvanometer by light transmitted through standard solutions, the value for K_1 can be found experimentally. In our studies K_1 had a value of 0.1565 where the Fe content of milk would be:

$$\text{mg. Fe/liter} = \frac{2 - \log G}{0.1565}$$

The procedure of assaying the iron content of milk is as follows: 100 ml. of milk is placed in a platinum crucible, evaporated and initially carbonized in an oven (open door) at 110°C. Carbonization is completed in a muffle furnace at dull red heat. The ash is taken up in 20% HCl, and filtered through Whatman's ashless paper no. 40. The residue is reignited in a muffle furnace, taken up in 20% HCl and filtered. The filtrates are combined, then boiled in a water bath for 20 minutes and the volume kept constant by addition of 20% HCl. After cooling, the filtrate is transferred to a 50-ml. volumetric flask and made up to volume 50 ml. with water. One milliliter of this is transferred to another 50-ml. volumetric flask and treated in the same manner as the standard solutions. As a test of the accuracy of the method, known amounts of iron were added to milk samples which had been analyzed and subsequent determinations made. The average iron recovery in a series of seven samples was 98% of the theoretical recovery.

This modification of Elvehjem's method appears satisfactory, but it is well to point out that the color produced with sulfocyanate varies depending on the dissociation of the complex iron salt (personal communication from Dr. C. A. Elvehjem). We concluded from other experiments that this difficulty is almost completely eliminated by the use of standard volumes.

MILK AND HEMOGLOBIN STUDIES

Eleven lactating Shoshone Indian women were picked at random and the assays of iron in milk were as follows: 1.72, 1.80, 1.76, 1.59, 1.84, 1.72, 1.70, 1.72, 1.79, 1.72, and 1.78 mg. Fe/liter. Three women were also studied following parturition and hemoglobin determinations carried out on their children. The iron content of milk of the three women under special study is presented in table 1.

TABLE 1
The iron content of breast milk.

SUBJECT I		SUBJECT II		SUBJECT III	
Date	Fe	Date	Fe	Date	Fe
1940	mg./liter	1940	mg./liter	1940	mg./liter
10/12	1.84	11/4	1.71	12/4	1.84
10/16	1.91	11/8	1.68	12/8	1.78
10/20	1.72	11/12	1.74	12/14	1.84
11/1	1.74	11/16	1.82	12/20	1.70
11/2	1.78	11/20	1.72	12/30	1.62
11/10	1.82	11/30	1.74	1/10/41	1.74
11/15	1.78	12/4	1.70		
		12/20	1.74		

The foregoing data indicate that the iron content of milk is a fairly constant one and is in the neighborhood of 1.72 mg./liter. In 1928 Peterson and Elvehjem ('31) determined the iron content of milk and found it to be 2.4 mg./liter. We determined the iron content of the milk of four normal, healthy white women and obtained the following values: 2.3, 2.4, 2.3, and 2.4 mg./liter. Thus it would appear that in normal, healthy women of our own culture the figure is somewhat higher than that for the Shoshone Indian, even though the milk from the former is far from sufficient in iron to prevent the so-called "milk anemia" from developing. On the other hand the dietary of the Shoshone woman, high in refined cereal carbohydrate and fat and low in meat and vegetable proteins and iron containing foods, might conceivably play a role in reducing the iron content of maternal milk. Furthermore, the method of assay used gave a figure for cow's milk (2.4 mg.

Fe/liter) similar to that reported by Peterson and Elvehjem. On cow's milk and special feeding formulae where the iron content of the milk was found to be on the average of 2.4 mg./liter, a series of five infants maintained an anemic state, whereas those which received a supplement of iron showed extraordinary increase in hemoglobin. Such findings in other people have been noted before but were repeated in this study of the Shoshone Indian in order to obviate any possibility of

GROUPS OF ARTIFICIALLY FED INFANTS

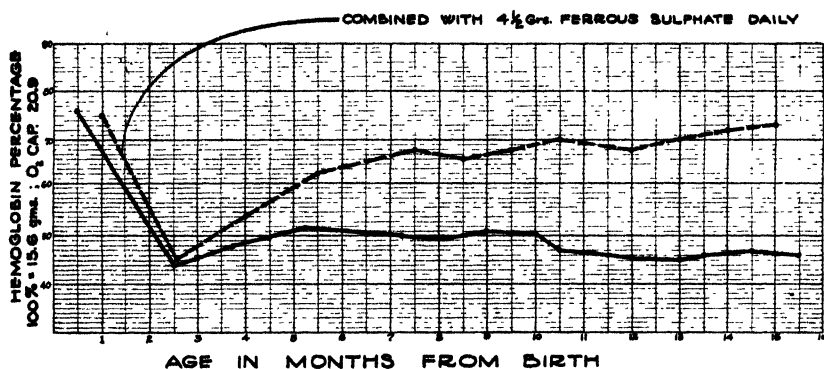


Fig. 1 The figure represents two groups of infants, both having manifestations of anemia. One group was placed on a feeding formula which had an iron content of 2.4 mg./liter. The other group received $4\frac{1}{2}$ gr. of ferrous sulphate daily. The first group continued to exhibit anemia, while the second group showed an appreciable increase in hemoglobin.

the anemia being due to other causes. Figure 1 represents two groups of five infants each where the limit of variation in each group in regard to hemoglobin content did not exceed 10%.

It would thus appear that iron is the limiting factor for hemoglobin formation in early infancy, all other things being equal. Hemoglobin determinations were also carried out on the children of the three mothers who were subjects of the milk iron study. The findings are reported in table 2.

From this table it would appear that the hemoglobin content at birth varies and the degree of red cell destruction and

anemia occurs at a variable period. No explanation for this is offered, especially in view of the fact that the iron content in the milk from the three mothers were within approximately the same range in content. Furthermore, the milk output in the three mothers did not differ appreciably (1440–1800 ml. daily).

Blood smears taken at weekly intervals from the children showed no unusual findings. Microcytes appeared during the fourth and fifth week, and from then on the blood picture was that of a secondary anemia.

TABLE 2

Hemoglobin content of blood in the three infants from the mothers studied.

INFANT FROM SUBJECT I		INFANT FROM SUBJECT II		INFANT FROM SUBJECT III	
Age	Hemoglobin	Age	Hemoglobin	Age	Hemoglobin
<i>Days</i>	<i>gm./100 ml.</i>	<i>Days</i>	<i>gm./100 ml.</i>	<i>Days</i>	<i>gm./100 ml.</i>
at birth	19.4	at birth	20.6	at birth	18.6
2	19.0	6	20.4	12	17.4
6	17.8	10	18.2	18	17.8
10	16.4	18	19.0	22	17.6
22	15.2	22	15.8	33	16.0
31	14.4	27	15.6	38	15.6
36	13.6	33	10.3	42	15.8
41	13.6	52	10.6	47	14.2
51	13.2	60	10.4	52	12.1
57	13.1	64	10.4	57	12.8
62	13.2	74	10.8	62	12.1
				67	11.4

DISCUSSION

The anemia resulting from an inadequate iron intake is significant in view of the fact that Rose and her associates ('30) found that an iron deficit occurred in a child who received 4.64 mg. a day. In such a case the iron output measured 5.74 mg., indicating a shortage in the food. Leichsenring and Flor ('32), Daniels and Wright ('34), Ascham ('35), and Porter ('41), in spite of some differences in their findings, advocate a liberal allowance in iron containing foods. On a milk regime the intake would be far from adequate (vide fig. 1) and inasmuch as the daily intake would never exceed 3 mg. from the total milk ingested, a low-iron anemia results.

Among Indians where many food habit deculturation processes have occurred, the tendency today is to breast feed the infant exclusively until such time as the child may eat of the food consumed by the rest of the family. Some Indians informed the investigators that in the past their parents used to chew food and then place this previously masticated food in the infant's mouth. Such a process was considered unhygienic by the invading culture and the practice was abandoned; it is, on the other hand, still continued by the Eskimo. One can assume that such a procedure might circumvent the effects of continued and exclusive milk feeding and thus obviate a low iron anemia.

The present problem is an interesting one and merits attention, for as the child continues in life (this is the subject of a separate communication) vicious circles are established wherein anemia, infection and certain avitaminoses mutually conspire against the body economy of these people. The problem is applicable to other Indian groups where continued breast feeding of an exclusive milk regime results in an anemia.

CONCLUSION

An anemia, secondary in character, is found among Shoshone Indian infants and is due to the continued and exclusive use of milk in their dietary. A modification of Elvehjem's method for determining the iron content of milk is introduced.

ACKNOWLEDGMENT

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BLOOD PLASMA ASCORBIC ACID VALUES RESULTING FROM NORMALLY ENCOUNTERED INTAKES OF THIS VITAMIN AND INDICATED HUMAN REQUIREMENTS ¹

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TWO FIGURES

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Ascorbic acid requirements for the human have been estimated over a wide age range by a variety of methods using differing criteria of adequacy and employing subjects with ascorbic acid deficiencies, subjects presenting other clinical problems, and subjects reported to be in a normal state of health. This latter group engaged in normal pursuits constitutes a sample of special value in establishing ascorbic acid requirements of individuals free from pathological symptoms.

The data which constitute present knowledge as to human requirements both in normal and pathological states have been obtained largely by urinary excretion studies, blood plasma analyses, and a combination of the two. Urinary excretion was early used as a means of evaluating human requirements. Refinement of techniques has made the measure of blood plasma ascorbic acid a rapid and valuable method for estimating ascorbic acid present for tissue use and adequacy of intake. Extensive use of this measure in population

¹Part of the data in this paper was included in a thesis presented to the faculty of the Graduate School of the University of Tennessee in fulfillment of the requirement for the M.S. degree, June 1942 by Harvey Lewis.

surveys has tended to fix relationships between plasma levels and intake within rather narrow limits. Simultaneous studies of blood and urine have been made especially in studies of threshold and saturation levels.

Whatever the method used the primary objective has often been to determine the daily intake of ascorbic acid necessary to maintain saturation. Saturation itself has been variously defined but the underlying concept seems to have been that the least amount that is in excess is bound to be sufficient. In such studies, fixed intakes have been ingested over periods extending from 6 to 28 days; these periods have been preceded by intakes of 100–200 mg. to insure against tissue lack, terminated by test doses of 400–600 mg., and often succeeded immediately by another period of observation on measured intake. The test doses as well as the preliminary saturation intakes are greater than those of the average diet or those recommended. The assumption is that the body quickly readjusts to a state of equilibrium with the lower levels which are the object of study.

In this laboratory a survey study on 345 students has shown only partial correlation between plasma ascorbic acid value and the calculated amount of ascorbic acid in the previous day's dietary. Experiments conducted with eight subjects for a 2-week period on high intakes, 200–400 mg., failed to produce consistent or similar saturation levels in the plasma for the group or for individuals. An extended study on a single subject alternating high and low intakes of the vitamin failed to show a regular or immediate response. These findings throw doubt on fixed relationships between plasma levels and intakes.

The present study was undertaken for the purpose of observing blood plasma ascorbic acid values when ascorbic acid intakes were those ordinarily encountered. Intakes were maintained at each level for periods long enough to indicate adjustment. The change to succeeding levels was made without interruption by large test doses. Continuous observation of plasma ascorbic acid values on fixed levels should indicate

the tissue status with respect to ascorbic acid by a decrease, a gain or a leveling off of these values.

EXPERIMENTAL

Experimental data were collected from four subjects in 1942 and from eight in 1943 during January, February and March. The study was made at four levels of ascorbic acid intake. A synthetic vitamin preparation² was given daily in amounts of 25, 50, 75 and 100 mg. These amounts were augmented by the ascorbic acid of the basal diet which was essentially that of Belser, Hauck and Storvick ('39). A composite sample of foods which contributed ascorbic acid was analyzed daily. In 1942, the diet furnished 10.2 ± 0.47 mg. daily; in 1943, 6.9 ± 0.36 mg. daily. Intakes were, therefore, 32-35, 57-60, 82-85 and 107-110 mg. In 1942, the food was analyzed by the method of Woessner, Elvehjem and Schuette ('39), originally used for milk. In 1943, a modification of the Loeffler and Ponting ('42) method was used.

Foods containing ascorbic acid were eaten in the same amounts by all subjects. A daily dietary record was kept for each individual and checked with Recommended Dietary Allowances (National Research Council, '43). The subjects omitted rich sources of ascorbic acid from their diets for 2 weeks before the beginning of the experiment. They remained on each level of intake for 2 weeks; data on the 85- and 110-mg. levels secured for a third week in the first study gave no further significant information.

Fasting blood samples were taken each morning and analyzed immediately by the Mindlin and Butler micromethod ('38). During the last half of the second study 24-hour urine samples were collected daily from three subjects. These were analyzed by the method of Evelyn, Malloy and Rosen ('38) using Bessey's ('38) method of correcting for turbidity and color. At the close of the experiment all eight subjects were given a test dose of 400 mg. synthetic ascorbic acid and 24-

² "Cebione" was generously supplied by Merck and Company.

hour urine samples were analyzed for excretion of the vitamin to check the subjects by the criterion of saturation.

RESULTS OF PLASMA ASCORBIC ACID STUDIES

Plasma ascorbic acid values of all subjects showed daily fluctuations at every level of intake. Daily values are shown (fig. 1) for three subjects, 6, 7 and 11, who were representative

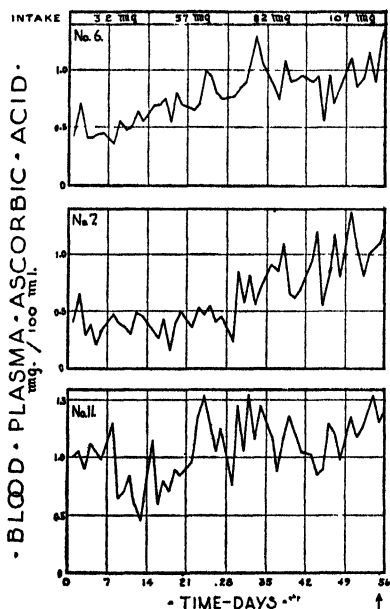


Fig. 1 Daily fluctuations of blood plasma ascorbic acid values for subjects 6, 7 and 11 at four successive levels of ascorbic acid intake. Arrow indicates intake of 400 mg. ascorbic acid.

of the group. The amplitude of the fluctuations and the time interval between successive high and low values tended to be quite regular and to a degree characteristic of the individual. On the 32- to 35-mg. intake level, successive upward swings gradually became less and downward swings dipped farther so that the general trend was a decrease in blood plasma values. As intake was increased this process was reversed. Two factors seemed reflected, rhythmic fluctuation and adjustment to different levels of intake.

An increase in intake did not immediately affect the fasting blood plasma ascorbic acid. Frequently the plasma value on the day following increased intake was decreased and was in general not typical of the intake but of the fluctuation. Values following the 400-mg. test dose at the close of the experimental period were within the range found on the 107- to 110-mg. intake. Adjustment to new levels came gradually. If weekly averages of daily plasma values are taken to indicate response to intake, definite trends can be observed (fig. 2). Holmes, Cullen and Nelson ('41) suggested that an average of a series of daily values gives a better estimate of the plasma ascorbic acid than single values taken at intervals.

The 32- to 35-mg. intake was characterized in all subjects by a decrease or bare maintenance of plasma levels. Individual differences did exist, however; extreme averages of 0.27 and 0.76 mg. % were found for the second week. This general decrease of plasma levels allowed subsequent changes with increased intake to take place uncomplicated by an accumulation of reserves.

The first week on 57-60 mg. generally resulted in plasma ascorbic acid values approaching those of the first week on the 32- to 35-mg. intake. By the second week all subjects showed increasing values with the exception of subject 5. Leaving subject 5 out of consideration, the extreme averages, 0.44 and 1.25 mg. %, would not normally have been expected on intakes identical for 4 weeks, yet the two represent the same dynamic picture, for this intake was satisfying normal body demands and furnishing a surplus which gradually appeared in the blood.

In eight of the twelve subjects the 82- to 85-mg. intake by the end of 2 weeks produced average plasma ascorbic acid values approximately equal to, or greater than those found later on the 107- to 110-mg. intake. For subject 5, this intake gave the first evidence of an amount great enough to supply body demands and to have an excess appear in the blood.

In the 1942 study no subject showed a rise in plasma ascorbic acid average due to the last 25-mg. increase in intake, while

in the 1943 study four of the eight subjects showed no rise. The other four subjects had not taxed the blood to the limit of its carrying capacity and at the end of 2 weeks at the 107- to 110-mg. level their plasma ascorbic acid averages were still mounting. After 8 weeks on identical ascorbic acid intake six of the eight subjects had blood plasma averages close to 1.0

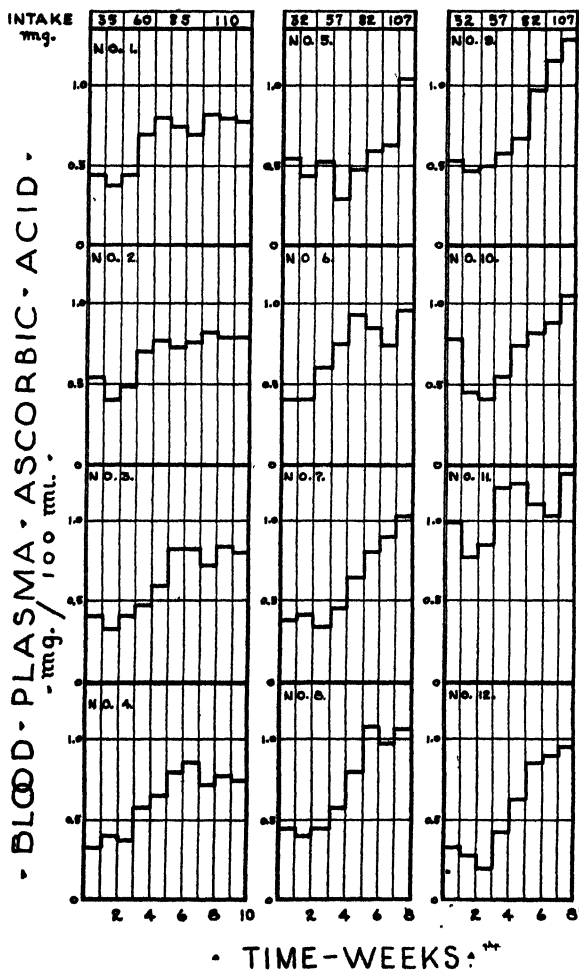


Fig. 2 Weekly averages of daily blood plasma values for each subject at four successive levels of ascorbic acid intake.

mg. %. In spite of this similarity of absolute values the behavior of individuals differed. One group was static and showed no tendency to alter plasma levels significantly. The other group was still building up the ascorbic acid content of the blood.

At the close of the experiment all subjects with the exception of 5, 9 and 10 were found to be saturated as judged by the urinary return of over one-half the test dose.

RESULTS OF URINARY EXCRETION STUDIES

Three subjects, 6, 7 and 11, were chosen for urinary excretion studies. During the first half of the experiment subject 11 had exhibited high plasma values, subject 7 low, while subject 6 gave values fairly typical of the group (fig. 1). Body weights of the three subjects differed. Subjects 7 and 11 were approximately the same age, while subject 6 was older.

Urine analyses were begun 2 days before the end of the 57-mg. intake and continued daily for the last 4 weeks of the experiment. Analyses were made on 24-hour samples collected after the first voiding in the morning. The results are shown in table 1. There was a daily fluctuation in excretion

TABLE 1

Average daily excretion of ascorbic acid for weekly periods at two levels of intake and retention related to body weight.

	PERIOD	AVERAGE INTAKE	AVERAGE EXCRETION, STANDARD ERROR	AVERAGE RETENTION	RETENTION PER UNIT BODY WEIGHT
	<i>wk.</i>	<i>mg./day</i>	<i>mg./day</i>	<i>mg./day</i>	<i>mg./kg.</i>
Subject 6	5th	82	22.8 \pm 2.8	59.2	...
Wt.—61.8 kg.	6th	82	30.5 \pm 4.8	51.5	0.83
Age—41 yr.	7th	107	38.0 \pm 3.2	69.0	...
	8th	107	51.0 \pm 2.0	56.0	0.91
Subject 7	5th	82	14.0 \pm 3.1	68.0	...
Wt.—66.4 kg.	6th	82	10.0 \pm 0.7	72.0	1.08
Age—23 yr.	7th	107	24.7 \pm 6.2	82.3	...
	8th	107	35.7 \pm 3.6	71.3	1.07
Subject 11	5th	82	25.8 \pm 3.4	56.2	...
Wt.—51.4 kg.	6th	82	25.9 \pm 2.1	56.1	1.09
Age—22 yr.	7th	107	39.5 \pm 6.6	67.5	...
	8th	107	49.9 \pm 2.0	57.1	1.11

as shown by the standard error, but weekly averages exhibited definite trends at the two levels of intake. Retentions were calculated for the second week at each level to permit adjustment to the 25-mg. increase of intake which appeared to be complete within 4 to 5 days. The saturation test made at the end of the experiment showed all three subjects to be saturated. Average retentions of ascorbic acid during the sixth and eighth week of experiment were in good agreement indicating that for these three subjects an 82-mg. intake was sufficient to supply normal body needs and provide an excess. An additional increase of 25 mg. was returned in the urine.

Retentions for subjects 6 and 11 were the same, while those for 7 and 11 differed. When related to body weight retention values for subjects 7 and 11 became 1 mg. per kilogram. For subject 6 who was older they were slightly lower. This finding is in keeping with reported data indicating high requirements for preschool children (Everson and Daniels, '36) and showing decreased concentration of ascorbic acid in the tissues of the human with age (Yavorsky, Almaden and King, '34). This retention or "utilization" of approximately 1 mg. ascorbic acid per kilogram of body weight approaches the lower values reported by others (Todhunter and Robbins, '40; Bryan, Turner, Huenemann and Lotwin, '41; Fincke and Landquist, '42) as the intake required to maintain saturation and was adequate to effect saturation in these three subjects.

DISCUSSION OF RESULTS

The trend of the data obtained during the 1942 and 1943 experiments was similar. Daily fluctuations in ascorbic acid values were a reality. They have also been reported by Storvick and Hauck ('42) and Roberts and Roberts ('42). Duplication of results was good; subject 2 of 1942 was subject 6 in the 1943 study, subject 3 was subject 7, and they exhibited similar values especially on the lower intakes. On the 32- to 35-mg. intake all average plasma ascorbic acid values were tending to decrease or had become stabilized. When the intake was increased to 57-60 mg. all subjects but one showed a

small surplus in the plasma which persisted throughout this period. In general, plasma values increased and reached a maximum during the 82- to 85-mg. intake. That all subjects might have reached a maximum at this level had the period been extended is open to question, but in the light of the urinary excretion data it is a possibility.

Single plasma ascorbic acid values or those taken at extended intervals give little information concerning the intake either as to absolute quantity or adequacy. The same values might be found at any two consecutive levels of intake; the extremes at any one level were frequently greater than the differences of weekly averages throughout the total range. Even weekly averages cannot be considered a satisfactory measure of intake; for example, those for the first 3 weeks were indistinguishable.

With increasing intakes of 32 to 110 mg., uninterrupted by large test doses, blood plasma ascorbic acid determined daily and for a long enough time to establish trends promises to give the clearest reflection of the system's requirements and adjustment to intake. If successive plasma ascorbic acid values are decreasing a deficit is being created. If they are increasing the system is utilizing what it needs and permitting a surplus to appear in the blood. If the values are definitely shown to be stabilized in the range characteristic of maximum blood levels the intake exceeds the requirements but the amount in excess cannot be judged.

The saturation test at the close of the 1943 study showed five of the eight subjects to be saturated on the basis of the return of over half the test dose. For subjects 5, 9 and 10 who were not, the adequacy of the intake to satisfy tissue needs is evidenced by the appearance of steadily increasing amounts of ascorbic acid in the plasma.

The urinary findings indicating adequacy at an intake of 1 mg. of ascorbic acid per kilogram of body weight when extended to trends shown by plasma averages of all subjects strengthen the relationship of intake to body weight. Subject 5, whose plasma values did not increase until intake rose to

82 mg., weighed 84.1 kg. Subject 11, whose plasma values started to rise on the 57-mg. intake and reached maximum on it, weighed 51.4 kg. All other subjects of intermediate weights, 55-69 kg., began to show plasma average increases on the 57- to 60-mg. level.

SUMMARY

1. A study of daily ascorbic acid requirements of the normal human adult has been made on twelve subjects on controlled, normally encountered ascorbic acid intakes of 32 to 110 mg., gradually increased without interruption by large test doses, over periods of 8 and 10 weeks.

2. The subjects were brought into equilibrium or slight deficiency as shown by plasma ascorbic acid averages which were slowly decreasing or only maintaining themselves. This was accomplished by an initial period on a 32- to 35-mg. intake of ascorbic acid.

3. Weekly averages of daily plasma ascorbic acid values gave a better reflection of the vitamin status of the individual than single determinations. Trends thus established showed the dynamic status of the individual with respect to intake.

4. Urinary excretion studies were made on three subjects for a 30-day period. "Utilization" values were shown to be close to 1 mg. per kilogram body weight. An intake of 1 mg. per kilogram of body weight was shown to increase plasma ascorbic acid values for all subjects studied and three subjects were shown to reach saturation on such a retention.

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ACTIONS OF BENZEDRINE AND PROPADRINE IN THE CONTROL OF OBESITY ¹

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ONE FIGURE

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Benzedrine and propadrine have been described as drugs which are useful in controlling body weight by numerous observers (Ulrich, '37; Lesses and Myerson, '38; Rosenberg, '39; Donley, '37; Schube, McManamy, Trapp and Myerson, '37; Myerson, '39; Fulton and Humphrey, '39; Ersner, '40), although these results have been denied, or the interpretations qualified, by others (Baker, '38; Dub and Lurie, '39; Cutting, '43). The usual explanation for such an effect is that these drugs control appetite. Other possibilities are an increase in energy expenditure from the well known excitant effects of benzedrine, modifications in carbohydrate metabolism, or impaired utilization of food through modification of the activity of the gastrointestinal system (Ulrich, '37; Lesses and Myerson, '38; Rosenberg, '39; Nathanson, '37; Davidoff and Reifenstein '37; Beyer, '39; Ersner, '40). A systematic study of these possibilities under laboratory conditions does not seem to have been carried out. Hence, it was thought worthwhile to determine if facts could be established which would help to explain the clinical effects, or lack of them.

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EFFECTS ON WEIGHT

Before the mechanism of action of benzedrine and propadrine in controlling obesity could be studied in the laboratory, it was necessary to demonstrate whether or not the loss of body weight was caused in animals as in patients.

Twenty white rats were fed 0.2 mg. of benzedrine in each gram of food for 3 weeks while living in revolving cages (Slonaker, '39). The growth was stopped and a slight decrease in weight occurred during the first week of medication, followed by a slow resumption of weight gain as tolerance developed. When the drug was withdrawn, there was a sharp increase in weight sufficient to make up for the loss, and failure to gain, during the period of drug-intake. These rats ran less than normally while medicated, so that increased exercise was not responsible for the effects on body weight. In another series with 7-day experimental periods, wide ranges of dosages of both benzedrine and propadrine were fed. While there were some irregularities due to individual variations in food intake and hence in drug ingestion, yet both drugs caused a decrease in body weight of up to 15% in 7 days. As little as 0.1% benzedrine caused a 9.2% loss, and 0.5% propadrine a 6.6% loss, indicating a rather marked effect in a low dosage range.

In another group 5 mg. per kilogram of benzedrine was injected subcutaneously twice daily for 8 days in five adult rats. The average weight dropped 6.5%. In other experiments to be described below where effects on weight were observed incidentally to other observations, large losses were also produced. Hence, these observations demonstrated that benzedrine and propadrine diminished the weight of white rats, when given in adequate doses either orally or by injection.

INCREASED ACTIVITY

One possible explanation of the weight loss was that the drug led to an increased energy expenditure.

It has been recently reported from this department (Schulte et al., '41) that benzedrine markedly increased spontaneous

movements as recorded in "jiggle cages", in doses of 0.3 mg. or higher per kilogram subcutaneously, and that propadrine also caused a slight stimulation but only in much higher doses. However, in the experiments above, propadrine produced substantially the same loss of weight as benzedrine, which would be inconsistent with the markedly different potency of these two drugs as control stimulants.

When benzedrine was injected subcutaneously in doses of 0.3 mg. per kilogram or propadrine 20 mg. or more in rats in revolving cages, a marked increase in running occurred which sometimes lasted over 7 hours (Tainter, in press). Here also there was a large discrepancy between the effects of benzedrine and propadrine. The above experiments were short-term observations, and hence did not reflect the total 24-hour changes. Therefore, attention was directed to feeding experiments in which the drug was mixed in the diet, and activity throughout the entire 24 hours recorded.

In feeding experiments in revolving cages of 7 days duration, with 7-day control periods before and after, benzedrine caused a moderate increase in the daily revolutions in concentrations between 0.05 and 0.25 mg. per gram of food, which gave total intakes of approximately 10 mg. per kilogram daily. Higher doses depressed the activity. Propadrine increased the activity only in the range of 1.0 to 1.5 mg. per gram of food; lower concentrations were ineffective and higher ones depressed it. In a similar experiment on twenty rats, where the medication period was 21 days with correspondingly longer control periods, 0.2 mg. of benzedrine per gram of food gave an average intake of 18.1 mg. per kilogram daily. The activity decreased from about 7500 revolutions daily to 4700 on the sixth day, and then steadily recovered to normal as the rats developed a tolerance to the drug. The coincidental effects on body weight were described above. Obviously, the decreased activity could not explain the weight loss, since inactivity would conserve energy rather than the reverse.

These results, therefore, failed to demonstrate an increase in motor activity which might explain the loss of weight. However, since the criteria used were muscular movements of the animal, rather than oxygen consumption, it was conceivable that there might be changes in total metabolism which would not be reflected in activity determinations, but which could be measured from oxygen use over adequate periods of time. Therefore, the effects of benzedrine and propadrine on the oxygen consumption were studied.

OXYGEN CONSUMPTION

The first observations were on the effects of subcutaneous injections on the oxygen-use recorded hourly for 7 hours in the apparatus described by Van Winkle ('42). Three rats were studied simultaneously each day. Nine rats were used for the entire study, determinations being made repeatedly on each at intervals of 3 days or more, so as to compare the effects of the drugs in the same animals. In two control series, (table 1) all the rats were injected with physiological salt solution to measure the normal oxygen use after handling and injection of the animals. On other days various doses of benzedrine or propadrine were injected. The oxygen used each hour was observed, corrected to standard pressure and temperature, and calculated as milliliters of oxygen used per 100 cm.² of body surface. Since the peak effect was invariably during the second hour, the average values for that hour are given in table 1 together with the percentage changes. The total metabolism was also calculated for the 7-hour period, and the percentage changes from the drugs computed.

There were some irregularities in the averages from spontaneous variations in activity of the rats. However, the data demonstrated that benzedrine caused a marked increase in oxygen use. In fact, the total effects from the 8 and 10 mg. doses were not recorded because the metabolism was still elevated about 25% at the end of the 7-hour observation period. Propadrine did not change the oxygen use, the small de-

viations from the control values being well within the limits of variation. These results closely agree with the control values reported by Van Winkle, using the same apparatus, and also with his results with injection of benzedrine (Van Winkle, '42).

Benzedrine has been observed by others also to increase the oxygen use in acute experiments (Delaunois and Marri, '39; Kleitman, '39; Dill et al., '39), and there is no doubt that such must result from the marked muscular movements produced by adequate doses of the drug. However, conclu-

TABLE 1

Effect of subcutaneous injection of benzedrine and propadrine in white rats on the oxygen consumption in acute experiments.

DRUG	DOSE MG. PER K.	NO. OF EXPTS.	AVERAGE METABOLISM IN SECOND HOUR		AVERAGE TOTAL METABOLISM IN 7 HOURS	
			In ml. O ₂ per 100 cm. ³	Increase in percent of control	In ml. O ₂ per 100 cm. ³	Increase in percent of control
Control		18	84.5		572.3	
Benzedrine sulfate	1	9	101.4	20.0	609.0	6.4
	2	9	113.6	34.4	653.4	14.2
	4	12	138.0	63.3	759.7	32.7
	6	18	114.4	35.4	716.6	25.2
	8	9	129.7	53.5	811.3	41.8
	10	9	122.6	45.1	815.7	42.5
Propadrine	1	9	85.0	-5.3	618.9	8.1
Hydrochloride	5	9	84.7	0.2	555.7	-2.9

sions could not be drawn as to the mechanism of weight loss unless it could be shown that the total oxygen use was increased over a long period of time, rather than temporarily.

Accordingly, continuous observations for weeks were made on the total oxygen used by individual rats, when fed the normal diet as well as food containing various concentrations of benzedrine. The rats lived in the metabolism chambers continuously, and their total oxygen use was recorded. The chambers were opened briefly once a day to permit cleaning, weighing, and feeding, and the total daily oxygen was corrected for this short interval. Because room temperature fluctuations modify the oxygen use, the average tempera-

ture of the room was recorded, and periods of medication were alternated with periods of normal diet of several days each. The 24-hour oxygen volume was corrected to standard pressure and temperature and calculated as liters of oxygen in 24 hours per 100 cm.² body surface. The control values of each rat were used to calculate its regression line of metabolism against room temperature by the method of least squares. Deviations from this normal curve under the influence of the drug were calculated as percentage changes from the normal for that temperature. This is substantially the technique used by Van Winkle in his acute experiments (Van Winkle, '42). Since injections of propadrine had failed to change the metabolic rate even temporarily, these observations were made with benzedrine only.

In an individual animal under control conditions, daily deviations from the calculated normal metabolism averaged approximately 5%. To obtain a more general expression of the relationship of room temperature to oxygen use, the values for 100 control days on the seven rats were pooled and the regression line calculated. The formula was found to be $y = 3.70 - 0.51x$, where y is the oxygen used and x is the average room temperature. This covered the observed temperature range of from 19.6°C. to 27.1°C. within which limits the regression line was substantially linear. Because the grouped data were obtained from a relatively small number of animals differing in their individual rates of metabolism, the deviations from the regression line averaged 7% or ± 0.18 liter out of an average metabolism of 2.57 liters of O₂ per 100 cm.² in 24 hours. The standard error of the y value was ± 0.238 liter or 9.3%. Benzedrine was mixed in the food for a total of 90 days in concentrations between 0.01 mg. and 1.2 mg. per gram, giving intakes ranging between 0.63 and 99.6 per kilogram daily. The resulting metabolic rates are plotted in figure 1. There was no indication of an increased metabolism from benzedrine, except in rats ingesting the nearly fatal amounts of 50 mg. per kilogram per day or more.

This result is in marked contrast to the increase from even small single injections of benzedrine during the first hours after administration. If the lack of apparent effect were due to development of tolerance, there should have been the greatest metabolism from any given dose during the first day of administration with lesser effects as the tolerance was built up. However, regrouping the data according to the day of administration showed that the metabolic responses to a given concentration were as great, if not greater, on the second and third days as on the first day.

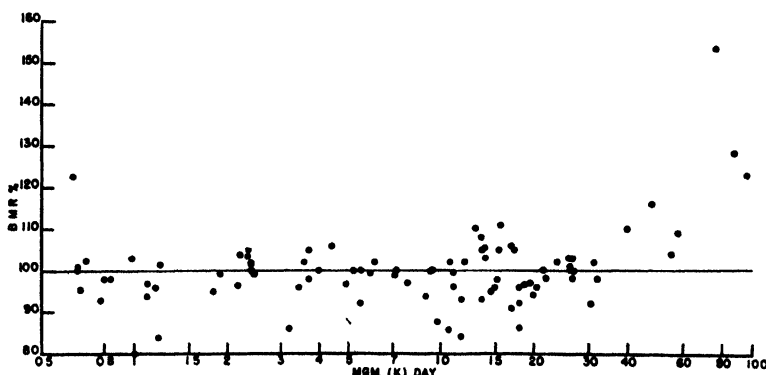


Fig. 1 Effects of various doses of benzedrine fed in the food on the oxygen use of white rats. Benzedrine doses are in milligrams per kilogram per day, and oxygen used in liters per 100 cm.² body surface per day.

These data show that, while benzedrine may stimulate oxygen use for approximately 6 hours after administration, the increased metabolism is compensated for by subsequent decreased energy output, so that the total 24-hour metabolism is not increased, except by doses approaching the fatal. This is similar to the observations of Searle and Brown ('38), who found that benzedrine redistributed the activity output, but did not modify its average 24-hour level.

PAIRED FEEDING EXPERIMENTS

Paired feeding studies were next carried out with benzedrine and propadrine. For each, five litter mate pairs of rats

were used, housed in individual cages. Normal food was given freely for 13 days and 21 days, respectively, before the drug was mixed in the diet, after which the food given daily to each control rat was equal to that eaten by the other member of the pair on the preceding day. Since the control rats did not always eat the food supplied them, they had a slightly smaller average food intake than those getting the drug.

TABLE 2

Results of adding benzedrine and propadrine to the diet. This represents two paired feeding series, in each of which five litter mate pairs were used. The figures in parentheses are the values for the control rats.

DAYS OF EXPT.	DRUG	CONCENTRATION OF DRUG IN FOOD	AVERAGE FOOD INTAKE	AVERAGE INITIAL WEIGHT	AVERAGE CHANGE IN WEIGHT
		mg./gm.	gm./rat/day	gm.	gm.
0-13	0	0	12.3 (12.7)	76 (76)	+ 37.8 (+ 33.4)
14-16	Benzedrine	0.6	9.2 (8.5)	113.8 (109.4)	- 6.8 (- 3.4)
17-48	sulfate	0.6	14.7 (12.5)	107.0 (106.0)	+ 39.6 (+ 56.2)
49-70		1.2	10.7 (10.2)	146.6 (162.2)	- 15.0 (+ 4.2)
71-92		2.4	9.1 (8.6)	131.6 (166.4)	- 11.6 (+ 15.1)
93-140		2.4	13.3 (11.5)	120.0 (181.5)	+ 21.0 (- 0.5)
0-11	0	0	8.2 (7.6)	38.0 (37.4)	+ 35.4 (+ 32.4)
12-21	0	0	10.0 (10.4)	73.4 (69.8)	+ 26.8 (+ 31.4)
22-30	Propadrine	2.4	9.2 (8.9)	100.2 (101.2)	+ 7.6 (+ 8.2)
31-40	hydrochlor-	2.4	10.7 (10.8)	107.8 (109.4)	+ 11.0 (+ 18.2)
41-59	ide	2.4	10.3 (10.3)	118.8 (127.6)	+ 16.8 (+ 30.6)
60-103	0	0	13.1 (12.5)	135.6 (158.2)	+ 57.2 (+ 51.0)

In the benzedrine series during the control days before medication, the gain was nearly equal on an almost equal food intake (table 2). For several days after 0.6 mg. benzedrine per gram was added to the food, the food consumption dropped sharply, and the medicated rats lost 6.8 gm. in weight. Their controls consumed even less food but lost only 3.4 gm. in weight. As the rats developed tolerance to the appetite effects of the drug, the food consumption increased, but the medicated rats gained less than did their unmedicated pairs, even though their net food intake was greater. This same sequence was reported each time the

benzedrine concentration in the food was increased. During the entire period, the medicated rats ate an average of 12.4 gm. as compared to the 11.0 gm. of their mates, but only gained an average of 27.2 gm. as compared to 71.6 gm. for the others. Obviously the medicated rats were much more affected than could be accounted for by simple diminution of food intake.

In the propadrine series there was a control period for 3 weeks before medication was started (table 2). There was a sharp decrease in the food intake to 6 gm. in the beginning of propadrine administration, but the appetite quickly returned, so that the average decrease in food intake during the first 8 days of medication was only to 9.2 gm. from the control of 10.0 gm. The differences in weight gain were not as marked with this dose of propadrine as with the benzedrine, but the same qualitative effects were present. Thus on an average food intake of 10.2 and 10.1 daily respectively, the medicated group gained an average of 25.4 gm. as compared to 57.0 gm. for the controls.

The mechanism by which this differential effect is brought about is uncertain. The most probable one is that the doses of benzedrine ingested were high enough to increase the metabolic level, since they were possibly in the stimulant range (figure 1). However, this thesis is difficult to sustain, since propadrine apparently does not increase metabolism nor activity in a manner similar to benzedrine. But, on the other hand, the changes from propadrine summarized in table 2 are so small as to be of uncertain probative value. If the energy metabolism of the medicated rats is not increased over the normal, the converse may occur, due to control rats having a decreased metabolism from the partial starvation. To decide between these hypotheses would require paired feeding experiments in metabolism chambers, which, unfortunately, could not be carried out at present. This phase of the study, therefore, must be deferred until some future time.

EFFECTS ON FOOD UTILIZATION

Because of the many reports that these drugs may relax the gastrointestinal tract and overcome spasm on the one hand, or cause stimulation on the other, the speed of passage of food through the gut was studied to determine if there were any changes which might impair the utilization or digestion of the food.

Twelve rats were selected for this purpose, each being used repeatedly at intervals of 4 to 7 days for a total of 180 tests, to compare the changes from the drug with numerous control observations. Food was removed from the cages the afternoon before the test. The next morning each animal was put into an individual cage in which there was powdered food containing 0.24% carmine. The animal was allowed to eat this food for 1 hour, during which time between 1 and 5 gm. were usually consumed, and then the colored food was replaced by normal diet. The drugs were mixed in the colored food, in concentrations from 0.3 to 2.4 mg. per gram, or were injected subcutaneously at the time the colored food was withdrawn from the cage. The stools of each rat were collected as passed in test-tubes of 1% ammonium hydroxide solution. The ammonia leached the red carmine color from the stools, without breaking them down or extracting the other pigments, and thus afforded an easy way of detecting the time of appearance of the carmine. Each rat was observed continuously until the stools became positive, and the time of passage of food mass thereby determined in hours from ingestion. Days of medication were alternated with control days to afford a reliable estimate of the normal times.

Inasmuch as the rats did not eat the same amount of food each day, it was necessary to establish whether the volume consumed would affect the passage time. Therefore, in control experiments, predetermined amounts of food were fed covering the range of amounts ingested during medication, and the normal times of passage averaged for different levels of food intake. At no level was there a significant deviation from the average of the entire series, nor was there any

tendency for a progressive change of speed with differences in amount of food ingested. Hence, the amount of food eaten did not affect the time required for it to pass through the gastrointestinal tract.

The average control passage time was 5.7 hours. When benzedrine was mixed in the diet, the animals receiving up to 5.0 mg. per kilogram, showed no change in the time. In the next group, where the average dose was 7.0 mg. per kilogram, there was a small but definite slowing to an average of 6.9 hours. This increased progressively with increase in dosage until at about 100 mg. per kilogram, there was apparently complete ileus for about 14 hours.

This slowing of peristalsis might be either a local effect of the drug directly on the wall of the gut, or a systemic action after absorption. Accordingly, in another series of rats, the benzedrine was injected subcutaneously instead of being fed. One-half mg. prolonged the time definitely, while 1.0 mg. and 2.0 mg. per kilogram increased it to about 11 hours. Similar observations with propadrine showed that it had at least as great an effect as the injected benzedrine, using doses of 0.5 and 1.0 mg. per kilogram subcutaneously. Obviously the slowing of peristalsis was much greater from injection of a given dose than from ingestion in food, which disposes of the possibility of there being a significant local effect on motility. Probably a part of the inefficiency of oral administration arises from delayed absorption as a result of the vasoconstrictor action of the drug on the intestinal mucosa.

The slow passage of food through the gut was undoubtedly accompanied by weak and inefficient peristalsis, and hence possible incomplete mixing with digestive enzymes and poor digestion. However, there was the opposite possibility that the prolonged time might have afforded a sufficiently longer interval for the completion of the chemical changes that there was no impairment of digestion and absorption. Evidence on this could be obtained by weighing the stools to see if there were a change in either direction which might throw light on these possibilities.

At this time, observations were being made of the effects of benzedrine on voluntary activity in rats, the results of which are being published separately (Tainter, in press). Since food intake, etc., were being measured, the stools of twenty rats were collected and weighed each day after drying at 110°C. If there were any undigested residue it would have increased the weight of the stools. During the 25 control days before medication, the dry stools weighed an average of 11.4% of the food intake. Benzedrine, 0.2 mg. per gram of food, was added to the diet giving an average dose of 18.1 mg. per kilogram daily, a dose definitely effective in changing intestinal motility. The dry weight of stools during the 21 days of this medication averaged 10.4% of the food intake. During the post medication (control) period of 22 days the weight of the dry feces was 10.3% of the food consumed. For the combined control periods, the average of 10.8% for the dry weight of the feces agreed closely with the 10.4% for the benzedrine-period. Hence, there was no evidence that the delayed passage of food through the gut was associated with changes in digestion or assimilation. Attention was, therefore, directed to the effects of benzedrine on food intake.

EFFECTS ON FOOD INTAKE

Data were available in many of the preceding series of experiments on the effects of benzedrine and propadrine on food intake or appetite. In a previous study (Tainter, in press) the average food eaten during 3 weeks of benzedrine and propadrine administration was compared with the corresponding non-medicated control periods. It was proved that any impairment of appetite was not due to the bitter taste of drugs in the food, since addition of other bitters did not decrease the appetite, but rather increased it, if anything. The average food intake for the twenty rats in the week preceding the drug was 93.8 gm. per kilogram daily. On an average daily dose of benzedrine of 15.4 mg. per kilogram during the first week, the food intake was decreased to an average of 77.3 gm. per kilogram daily. Thereafter a toler-

ance to the appetite effects of the drug developed so that, during the second week, the food intake had returned to within the normal range.

At first the average decrease in food eaten was 2.3 gm. per rat daily, which was accompanied by an average daily loss of body weight of 0.5 gm. The food had an energy value of 3.5 calories per gram, so the total deficit in caloric intake was 8.05 calories a day. Since animal fat has a caloric value 9.4 per gram, this decrease in caloric intake could have been made up by burning 0.85 gm. of the rat's fat, which, since fatty tissues are approximately 15% water, would have caused a loss of weight of 1.0 gm. a day. It is seen that the decrease in food intake was adequate to explain the observed loss of weight.

In another series, five rats were injected twice daily with 5 mg. of benzedrine per kilogram for 8 days, and lost an average of 15.6 gm. each. The average control food intake was 15.5 gm. a day, which the drug-injections decreased by 4.9 to 10.6 gm. a day. Such a decrease in energy intake could have been replaced by burning 17.1 gm. of fatty tissue. This checks well with the observed loss of weight of 15.5 gm. so that the diminished food intake would appear to be an adequate explanation of the loss of weight in this series also. It might be pointed out that, since the benzedrine was injected in these experiments, the effects on appetite were systemic rather than local on the gut before absorption.

The most extensive series of observations in which food intake and energy exchange could be correlated were in the oxygen-use studies where the rats lived in the metabolism chambers. Those rats with metabolism increased by the feeding of benzedrine (fig. 1) were eliminated from the present calculations. There remained twenty-two control periods and twenty-one medication periods on seven rats, totaling 89 days and 77 days, respectively. In the non-medication periods, the rats ate an average of 10.16 gm. a day and gained 1.22 gm. daily. During the drug-periods the food intake was decreased to 7.40 gm. per day, and an average of

1.38 gm. weight was lost daily. The benzedrine obviously decreased the food intake in the same way as in the other series described above. Calculation of the weight equivalent of the deficit in food intake accounted for a loss of only 1.21 gm. a day, if pure body fat were being used up. However, the net decrease in body weight was really 2.60 gm. a day, which can be accounted for satisfactorily if the rats were burning also some carbohydrate or protein, or had small shifts in water balance, or both.

Therefore, it appears that the observed changes in body weight in these various series of experiments can be explained by the measured changes in food intake, even when the doses of the drugs administered were so low as not to increase the energy output in spontaneous activity, or the total oxygen use. The effects of benzedrine and propadrine on appetite thus appear to be the primary explanation of the weight reducing power of these drugs.

SUMMARY AND CONCLUSIONS

1. Benzedrine and propadrine cause a loss of body weight in white rats in a manner comparable to that reported for patients.

2. Energy output changes as measured in revolving cages do not explain the decreases in body weight observed. Direct calculation of the energy metabolism from oxygen use in both acute and prolonged experiments showed that, while benzedrine increased the oxygen consumption for about 6 hours after subcutaneous injection, it did not change the total oxygen used in 24 hours unless the doses approached the fatal. Subcutaneous injections of propadrine did not change the metabolism even temporarily.

3. Both benzedrine and propadrine slowed the rate of passage of food through the gastrointestinal tract. This was not a direct local action on the intestine before systemic distribution since the same effects were obtained after subcutaneous injection of smaller doses. The slower passage through

the gut apparently did not modify appreciably the digestion or assimilation of food, since it produced no change in the dry weight of the stools.

4. In paired feeding experiments, medicated rats lost more weight than could be accounted for by decreases in food intake. These rats, however, ingested such a high dosage of the drugs as to bring them into the range where metabolic stimulation probably resulted. There was also the possibility of decreased energy utilization of the unmedicated control rats because of restricted food intake.

5. The food intake was markedly impaired by these drugs, especially during the first few days of medication when the weight changes were greatest. The decreased food intake was sufficient to explain the loss in body weight on the assumption that the energy of the food deficit was being made up mainly by burning body fat, or in some experiments by additional carbohydrate or protein, or by possible concomitant minor shifts in water balance.

6. The main result applicable to the clinical effects in obesity was this diminished food intake. It is noteworthy that a tolerance to the appetite effect of the drugs quickly developed, so that food consumption returned to normal after about a week.

These results indicated that benzedrine and propadrine might be of greatest value in those cases of obesity where control of the appetite was the most pressing need, and where the dosage could be kept low and duration of medication short to minimize the development of tolerance. Propadrine was weaker than benzedrine, but since it lacked the unpleasant central excitant effects of the latter, might well be used first in those patients where this type of treatment is desired. These results, taken as a whole, indicate that benzedrine or propadrine can probably serve only as an adjuvant to the already well recognized and commonly employed measures in controlling obesity.

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STUDIES OF THE COMPARATIVE NUTRITIVE VALUE OF FATS

I. GROWTH RATE AND EFFICIENCY OF CONVERSION OF VARIOUS DIETS TO TISSUE ¹

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FIVE FIGURES

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There are conflicting reports in the literature as to whether various animal and vegetable fats have similar nutritive properties aside from their vitamin content. It is known that with the exception of a few fats having abnormally high melting points, all are equally well absorbed in human subjects, the coefficients of digestibility approaching 95% (Holmes and Deuel, '21; Deuel and Holmes, '22). The requirement for certain unsaturated fatty acids present in a number of vegetable oils has been well established (Burr and Burr, '30). It has also been postulated by Schantz, Elvehjem and Hart ('40) that butter fat possesses specific nutritive properties since they found greater growth of rats during the first 6 weeks following weaning when the diet consisted of a homogenized mixture of skimmed milk and butter fat than

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when cottonseed, corn, coconut or soybean oils were substituted for butter fat. These investigators believe that this stimulatory effect is traceable to some component of the fat itself rather than of the unsaponifiable residue. It could not be ascribed to the phospholipid (Schantz, Boutwell, Elvehjem and Hart, '40a). But the administration of the saturated non-volatile acids of butter fat brought about the greater growth, while the unsaturated and volatile acids were inactive (Schantz, Boutwell, Elvehjem and Hart, '40b). Boutwell et al. ('41) found that the stimulatory factor could also be produced by hydrogenation of the unsaturated fraction in butter, but not by employing a similar procedure on the unsaturated fraction of the vegetable oils. In their most recent report, Boutwell et al. ('43) have found that the superiority of butter is only evident when the carbohydrate is lactose, while superior growth occurred with corn oil when the diet contained dextrin, sucrose, glucose or starch. Boer ('41) has also postulated that butter fat contains a specific growth-promoting property since he was able to obtain better growth in rats with butter than with olive oil diets. Gullickson, Fountaine and Fitch ('42) have reported that butter fat, tallow and lard are better tolerated by young calves and cause better growth than corn, cottonseed, soybean and coconut oils when the fats are homogenized in skim milk.

However, Euler, Euler and Saberg ('41) obtained superior growth in rats on margarine diets as compared with butter diets over a 6-week period where the average gain on margarine was 96.7 gm. compared with a mean gain on the butter diet of 77 gm. Freeman and Ivy ('42) found no statistically significant differences in early growth of rats fed milk with coconut oil as compared with butter fat although the butter-fed rats were larger after 3 months. These results are diametrically opposed to those of Harris and Mosher ('40) who found more rapid growth of weanling rats on butter fat and of older rats on coconut oil. In view of these apparent contradictions the following experiments were undertaken to follow growth rate, efficiency of conversion of food calories

into body tissue, the comparative composition of the body tissues of such rats, the effect of flavor on food preference and a comparison of pregnancy and lactation on such diets. The latter topics will be dealt with in later reports.

EXPERIMENTAL

Three series of tests were made on a total of 250 male and 309 female rats. In series I, the growth of twenty-two and twenty-four male rats was followed over 6 weeks on the butter and margarine diets described below; also the growth of two groups of female rats consisting of twenty-eight and twenty-four in the first case and eighteen and nineteen in the second set was followed over a 6-week period on the butter and margarine diets respectively. In series II and III, approximately fifteen rats of each sex were used with diets made up of each of the following fats over a 12-week period: a butter fat or a whole butter, corn oil, cottonseed oil, a margarine fat or a whole margarine, olive oil, peanut oil, or soybean oil. In series III whole butter and margarine were used and the diets were flavored by the addition to the fats of 4 parts per million of diacetyl which is the level preferred by the rats (Deuel and Movitt, '44).

The diets employed are listed in table 1.

The diets were prepared weekly and stored in the refrigerator to prevent rancidity. Several days' supply was placed at one time in the food cups. By the use of a special type of hopper practically no spilling of the food occurred and the food consumption could be determined with considerable accuracy. Animals were weighed weekly. Also at 3 and 6 weeks, the left tibia was x-rayed with a McFedries dental x-ray apparatus, the rats being lightly anesthetized with ether during this procedure. The length of the bones was determined by reading the x-ray photographs with a cathetometer.

The animals used were from our stock colonies. The litters were equally distributed between the various diets; when more than two in a litter developed diarrhea during the first week, the whole litter was discarded. To avoid differences in growth

TABLE 1
Composition of diets used.

COMPONENT	DIET I	DIET II
Mineralized skimmed milk powder ¹	70.6	70.6
Added minerals (per 100 lbs.) ²		
MnSO ₄ · 7H ₂ O	3.455 gm.	
CuSO ₄ · 5H ₂ O	3.340 gm.	
Fe · C ₆ H ₅ O ₇ · 3H ₂ O	45.557 gm.	
Butter fat ³ or margarine fat ⁴	29.4	
Vitamin D concentrate (per 1000 gm. fat) ⁵	801 mg.	
Butter fat, ⁶ margarine fat ⁷ , or vegetable oils supplemented as follows per 1000 gm. fat		29.4
Vitamin A: carotene ⁸ (40% of total A)	8 mg.	
vitamin A concentrate ⁹	133 mg.	
Vitamin D: viosterol ¹⁰	801 mg.	
Vitamin E: α tocopherol ¹¹	10 mg.	
Diacetyl ¹² or commercial butter flavor ¹³	4 mg.	

¹ Challenge brand prepared by roller process.

² Calculated to give 1.5 mg. Fe and 0.15 mg. each of Cu and Mn per 8 gm. of food.

³ Challenge brand.

⁴ Fortified with 7500 I.U. of vitamin A per pound.

⁵ 15,000 U.S.P. XI units per gram. Calculated to furnish 28.2 units of vitamin D per 8 gm. of food.

⁶ In series III where whole butter or whole margarine was used, these were increased so that the same amount of fat was present.

⁷ Fortified with 9000 I.U. per pound.

⁸ S.M.A. 90% beta and 10% alpha carotene. This gives 18.8 μg. or 31.4 I.U. per 8 gm. of food.

⁹ Kindly furnished by the Atlantic Coast Fisheries. Potency was 150,000 I.U. per gram or 45 I.U. per 8 gm. of food.

¹⁰ Calculated to give 18.8 U.S.P. XI units per gm. of food.

¹¹ Calculated to give 23.5 μg. per 8 gm. of food.

¹² Obtained from Larkin and Co., Buffalo, N. Y.

¹³ B F A obtained from Verley Products Corporation, 1621 Carroll Ave., Chicago, Ill.

ascribable to environmental conditions, the animals on the various diets were so located as to prevent any segregation according to diet.

The butters for the experiments in series II and III were of highest quality obtained in equal amounts from Minnesota, Wisconsin, Texas, Oregon² and California. The margarine,

² From farm of Oregon State College through courtesy of Dr. J. S. Butts.

in which the fat was a hydrogenated cottonseed oil, was purchased on the open market in New York, Chicago and Los Angeles. These samples were forwarded by express packed in solid carbon dioxide. For series II the butter and margarine were melted, the water and protein allowed to settle and the fat filtered out and mixed to give a homogenous sample. For series III, they were softened sufficiently to be mixed satisfactorily. The oils were obtained directly from the producer and shipped to us by express.³ They were stored in the refrigerator at approximately 38°F. in air tight containers in the dark, which procedure kept them fresh during the course of the experiments. Inasmuch as the rat is very sensitive to even slight evidences of rancidity in fats, any specimen of fat or oil which gave positive organoleptic evidence of rancidity was not used in this investigation. The free fatty acid content, saponification number, iodine number, and thiocyanogen number of the fats were also determined. These were normal except that the free fatty acid content in the olive oil samples were especially high, being 2.20 and 4.37% in the samples used in series II and III respectively although they possessed excellent flavors.

RESULTS

The growth rate of the male and female rats receiving the butter or margarine diets (diet I) over a 6-week period is illustrated in figure 1.

The growth of the rats on the various diets in series II and series III was similar for the first 6 weeks. Figures 2 and 3 give the average gain of male and female rats respectively on the seven different fats over a 6-week period. Figure 4 gives the average total gain of the rats in series II and series III after 12 weeks on the respective diets.

³ Cotton and peanut oils from Southern Cotton Oil Co., Savannah, Ga.; corn oil from local distributor, Corn Products Refining Co., soybean oil from Staley Manufacturing Co., Decatur, Ill., and olive oil from Neapolitan Olive Oil Products, Los Angeles.

The average tibia lengths of the rats in series III are given in figure 5 for 3 and 6 weeks on each diet. Similar results were obtained on series II for 6 weeks but no measurements were made in this series at 3 weeks.

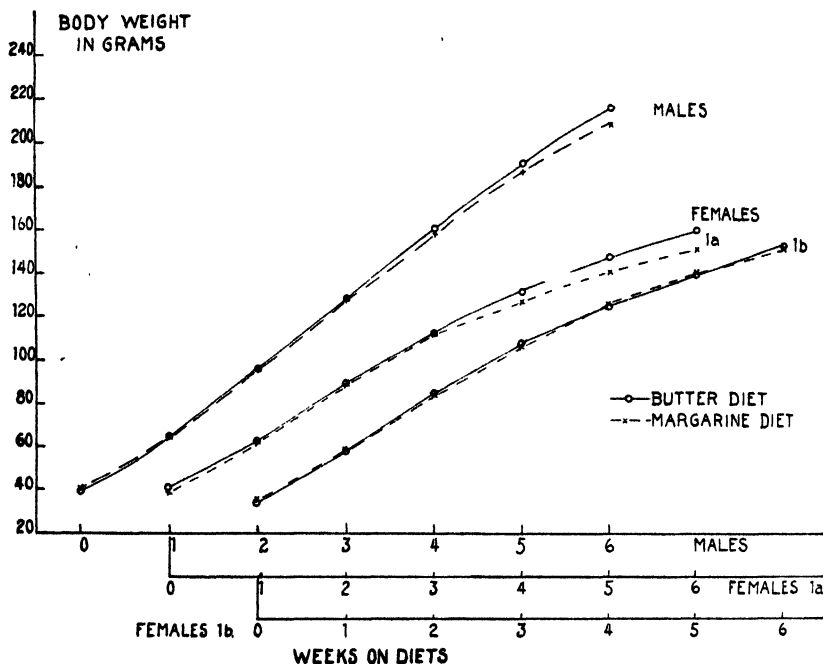


Fig. 1 The rate of growth of male and female rats on diets of mineralized skimmed milk powder mixed with butter fat or margarine fat. The following number of rats were used in the butter and margarine tests respectively: males 22 and 24; females in 1a, 28 and 24, and in 1b, 18 and 19.

The efficiency of transformation of the various diets to body tissue during the first 6 weeks is illustrated in table 2. Because of the slowing down of growth after that period, calculations of these ratios beyond that point are not included. Since the ratio of total food consumed to gain in body weight approaches infinity when the increase in weight approaches zero, we have omitted from the averages all experiments where the growth was 10 gm. or less. Practically no experi-

ments had to be dropped for this reason except with the females on fifth and sixth weeks. However, a clearer picture is probably obtained in the second average where all tests are included. This was obtained by dividing the total food consumed by all rats for the 6-week period by the total gain in weight of these animals over this interval.

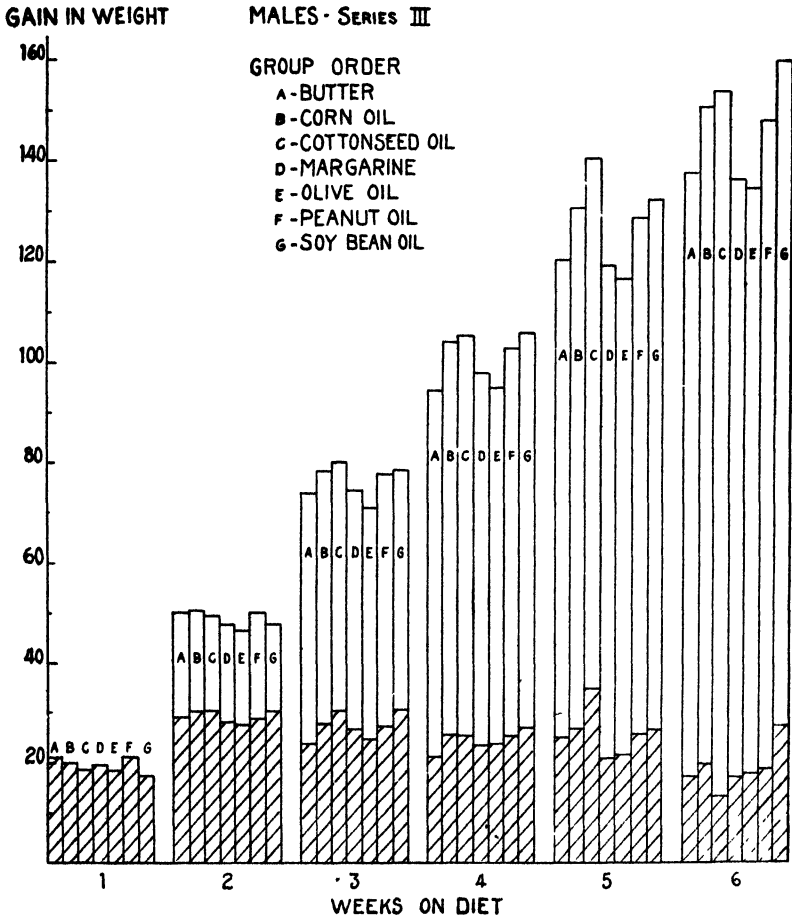


Fig. 2 The shaded portion represents growth of male rats for each week while the unshaded portion represents total growth from initiation of the experiment. The following number of animals were employed: butter (13), corn oil (13), cottonseed oil (14), margarine (13), olive oil (13), peanut oil (13), soybean oil (13).

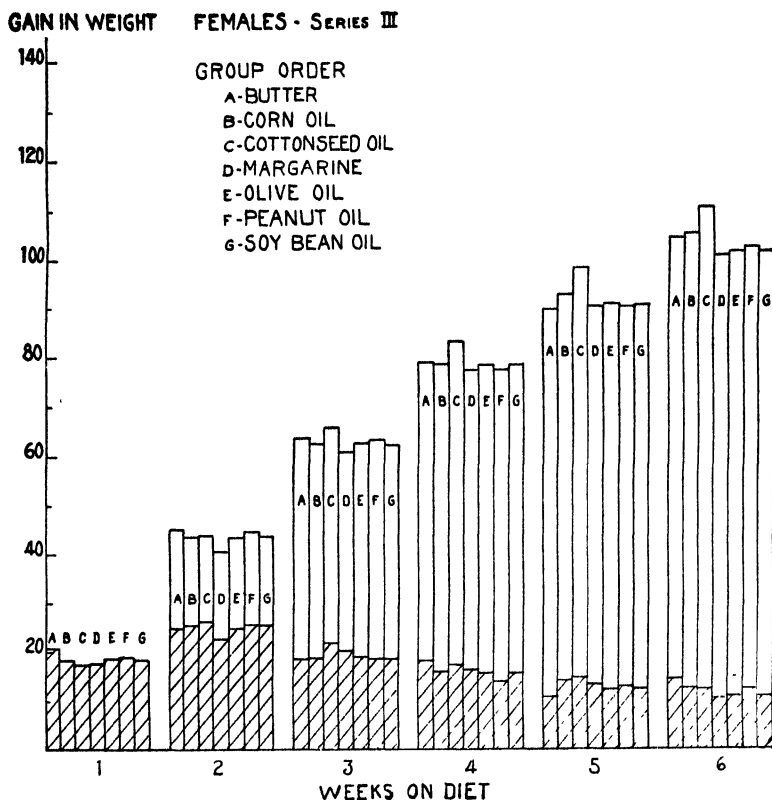


Fig. 3 The shaded portion represents growth of female rats for each week while the unshaded portion represents total growth from initiation of experiments. The following number of animals were employed: butter (15), corn oil (16), cottonseed oil (15), margarine (15), olive oil (13), peanut oil (15), soybean oil (15).

DISCUSSION

No differences were found in the rate of growth when weanling rats were placed for a period of 12 weeks on diets composed of mineralized skimmed milk powder, vitamin supplements and the different fats under investigation. The gains in weight of the male rats in series III at 3, 6 and 12 weeks were as follows: 3 weeks, butter 73.9, corn oil 78.1, cottonseed oil 79.7, margarine 74.3, olive oil 70.8, peanut oil 77.2 and soybean oil 78.4 gm.; 6 weeks, 137.3, 150.2, 153.6, 137.5, 134.0,

147.2 and 159.2 for the groups on the different diets respectively; 12 weeks, 225.5, 230.3, 230.6, 205.0, 206.5, 228.0 and 243.1 gm. With the female rats in the same series, the results were: 3 weeks, 63.8, 62.5, 65.8, 60.8, 62.6, 63.2 and 62.4 gm.;

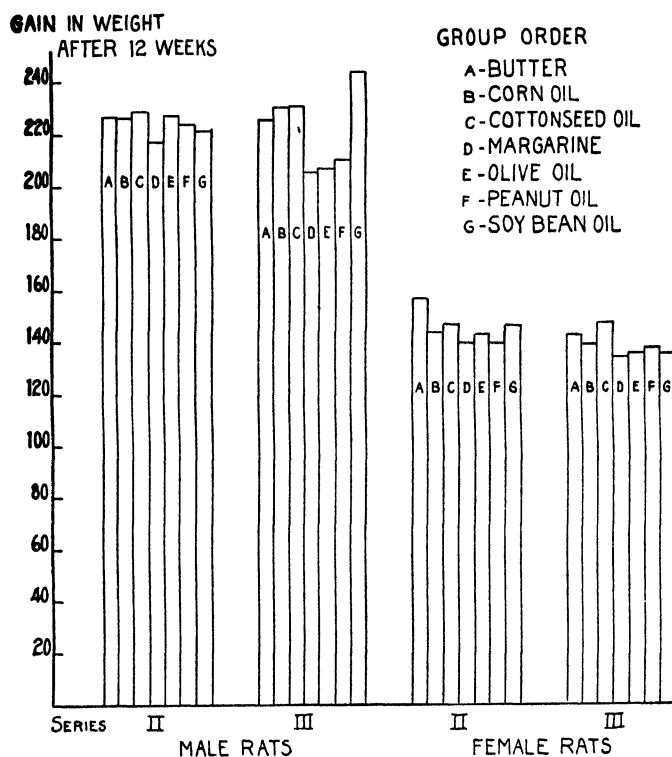


Fig. 4 The total gain of male and female rats in series II and III on the diets containing the various fats over a 12-week period. The following number of rats were employed: male rats (series II and III respectively — butter (12, 13), corn oil (14, 13), cottonseed oil (14, 14), margarine (12, 13), olive oil (16, 13), peanut oil (13, 13), soybean oil (13, 13); female rats — butter (15, 15), corn oil (14, 16), cottonseed oil (15, 15), margarine (18, 15), olive oil (12, 13), peanut oil (17, 15), soybean oil (17, 15).

6 weeks, 104.1, 105.0, 110.2, 100.5, 101.1, 102.3 and 101.3; 12 weeks, 142.4, 137.0, 147.2, 133.7, 134.8, 136.9 and 134.3 gm. In no single case was the average growth of the rats on the butter diet the highest. In the tests on male rats, it was sixth

at 3 and 6 weeks and fifth at 12 weeks. With the females the butter group was either second or third. However, in only a few cases were the differences statistically significant. Based on a greater value than 3.00 being significant for the ratio of mean difference to standard error of mean difference, the

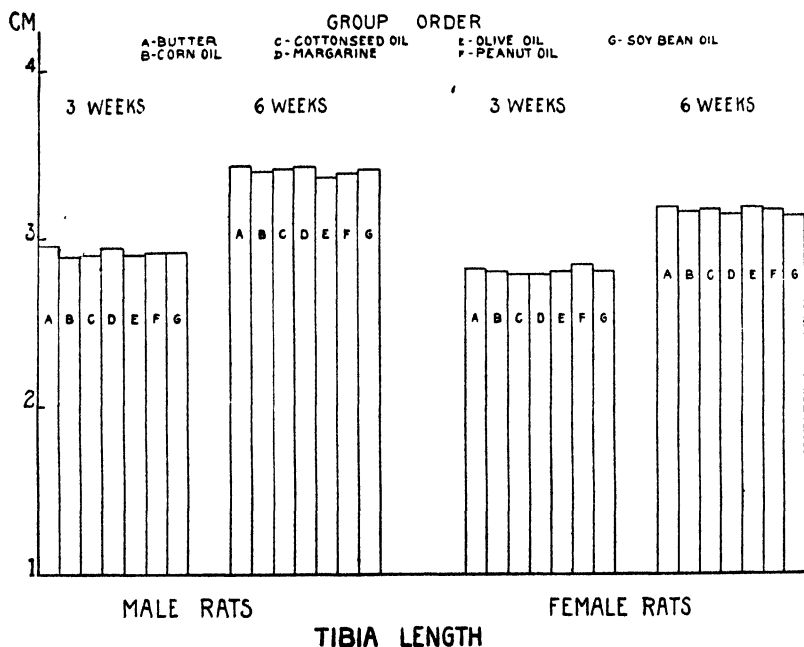


Fig. 5 The tibia length in centimeters for male and female rats after 3 and 6 weeks on various diets. The averages are for the following number of animals: male rats (3 and 6 weeks respectively) — butter (12, 13), corn oil (12, 10), cottonseed oil (13, 12), margarine (14, 12), olive oil (13, 12), peanut oil (13, 12), soybean oil (13, 9); female rats — butter (14, 12), corn oil (16, 13), cottonseed oil (15, 13), margarine (15, 13), olive oil (13, 11), peanut oil (15, 13), soybean oil (15, 13).

weights between the sixth and tenth weeks of the male rats receiving the soybean oil diet are statistically higher than those of the rats receiving butter, margarine or olive oil in the diet. On the other hand, the females on soybean oil did slightly poorer than the rats on the butter diet; however, in the tests with females there is no significant statistical difference between any fats at any period of the study.

TABLE 2

The ratio of food intake to gain in weight.

DIET	1ST WEEK	2ND WEEK	3RD WEEK	4TH WEEK	5TH WEEK	6TH WEEK	MEAN AVERAGE	TOTAL FOOD CONSUMED: TOTAL GAIN IN WEIGHT ¹
Male rats								
Butter ²	1.44 (15)	1.75 (15)	2.60 (14)	2.86 (13)	2.86 (15)	3.74 (12)	2.54 ...	2.54 ...
Corn oil	1.44 (15)	1.55 (15)	2.23 (15)	2.66 (15)	2.63 (14)	3.65 (14)	2.36 ...	2.35 ...
Cottonseed oil	1.38 (14)	1.51 (15)	2.01 (15)	2.63 (15)	2.63 (15)	3.24 (13)	2.23 ...	2.22 ...
Margarine ²	1.50 (15)	1.71 (16)	2.38 (16)	2.82 (16)	3.16 (14)	3.61 (13)	2.53 ...	2.38 ...
Olive oil	1.36 (13)	1.59 (15)	2.22 (15)	2.60 (15)	3.31 (15)	2.86 (11)	2.33 ...	2.29 ...
Peanut oil	1.40 (15)	1.69 (15)	2.25 (15)	2.55 (15)	2.67 (15)	3.66 (14)	2.37 ...	2.33 ...
Soybean oil	1.51 (13)	1.57 (15)	2.02 (15)	2.50 (14)	2.51 (14)	2.96 (15)	2.18 ...	2.17 ...
Female Rats								
Butter ²	1.47 (14)	1.73 (15)	2.95 (14)	3.65 (14)	4.09 (7)	4.30 (11)	3.03 ...	2.78 ...
Corn oil	1.55 (14)	1.86 (15)	2.82 (14)	3.48 (14)	4.36 (14)	4.41 (10)	3.08 ...	2.95 ...
Cottonseed oil	1.54 (15)	1.67 (15)	2.61 (15)	3.31 (15)	4.02 (14)	4.66 (10)	2.97 ...	2.71 ...
Margarine ²	1.67 (15)	1.79 (13)	2.50 (14)	3.23 (14)	4.04 (10)	4.58 (7)	2.97 ...	2.90 ...
Olive oil	1.54 (14)	1.78 (14)	2.43 (14)	3.48 (11)	3.99 (9)	4.56 (6)	2.96 ...	2.70 ...
Peanut oil	1.63 (15)	1.73 (15)	2.87 (15)	3.40 (11)	3.99 (11)	4.27 (8)	2.98 ...	2.80 ...
Soybean oil	1.52 (14)	1.69 (15)	3.05 (14)	3.40 (13)	4.16 (10)	4.87 (7)	3.12 ...	2.95 ...

The figures in parentheses represent the number of animals in the average.

¹ Experiments on all rats in these tests. Total food consumption of each group for 6 weeks divided by total gain in weight for the same rats over entire period.

² Corrected for water content of diet.

The uniformity in growth for the first 6 weeks on diets containing either butter fat or margarine fat is further emphasized in the results on series I. These data are also confirmed by series II. With male rats the gains at 3 weeks were 80.7 (butter fat), 84.7 (corn oil), 85.8 (cottonseed oil), 75.6 (margarine fat), 79.3 (olive oil), 83.8 (peanut oil), 82.1 (soybean oil) and at 6 weeks 147.6, 156.7, 160.0, 144.4, 152.7, 152.1 and 149.7 gm., respectively. In the tests on female rats in this series the cottonseed oil group was slightly higher at 3 weeks, the increases in weight being 72.8, 69.3, 74.0, 68.5, 71.3, 68.8 and 70.5 gm. respectively; at 6 weeks the butter group led but no differences were significant. The values at this period were 116.9, 108.7, 113.0, 105.0, 110.2, 105.9 and 110.4 gm., respectively. At 12 weeks in this series as shown in figure 4, the results on the males were identical; the levels on the butter group were slightly higher but in no case significantly different than any other groups from a statistical standpoint, in the case of the females.

That the growth of the rats on the various diets represents a real increase in size is demonstrated by the fact that a similar bone growth obtained. The length of the tibia in the various groups was found identical after 3 and 6 weeks on the diets in series III (fig. 5). It is further confirmed by the results at 6 weeks only in series II where the tibia lengths in centimeters were found to be as follows for the seven diets: male, 3.30, 3.36, 3.39, 3.28, 3.37, 3.44, 3.35 and females, 3.14, 3.13, 3.16, 3.06, 3.16, 3.10 and 3.14.

Moreover, further evidence of the similarity in nutritive value of the various fats is obtained from the results of the efficiency of transformation of the food to body tissues (table 2). Identical values were obtained at all periods and there is no case where the discrepancies noted by Schantz et al. ('40) were found.

There are several explanations as to why these results and those of Schantz et al. ('40) are contradictory. We mixed the fats with skimmed milk powder rather than with the liquid skimmed milk, and this renders a more uniform diet available

to the growing rat throughout each day. In their last study, Boutwell et al. ('43) indicate that in their earlier experiments the fat "tended to separate out of the liquid diet so that the rats ate an abnormally high fat diet soon after feeding time, leaving nearly skim milk to eat the rest of the day". Obviously with such irregularities in composition of the milk, one cannot accept as valid the differences in efficiency of transformation of the various skimmed milk diets to body tissue reported by Schantz et al. ('40).

Secondly, in our tests, the food consumption was approximately the same in all groups. Since growth is so closely related to the intake of nutrient materials, it is obvious that decreased food intake per se may be the cause of decreased growth. Boutwell et al. ('41) have indicated that the lower growth rate of animals on the corn oil diet as compared with those on the butter or butter fat fractions was associated with a smaller daily consumption of food. However, they indicate that the increased appetite and greater palatability are the attributes of a superior food. We believe that there are additional factors involved in appetite and food consumption. Although we have found that animals in general prefer a butter diet to one containing the other vegetable fats, they will also overwhelmingly prefer a diet of peanut oil with commercial butter flavor to one which is unflavored. Obviously, it is flavor rather than nutritive value which guides the choice here.

Thirdly, in the original paper of Schantz-et al. ('40), there are wide variations in starting weight favorable to the butter group. In the series of male rats the cottonseed and soybean oil groups weighed on an average only 32 gm. compared with 42 gm. for those on butter; with the females the comparative groups weighed 30 and 39 gm., respectively. In our experiments in series I, the ten animals lowest in weight at the start of the tests (32.6 gm.) gained only 103.4 gm. while the ten heaviest weanling rats (42.8 gm.) increased by 130.8 gm. over a 6-week period. In the tests reported in the present paper

the average weights of the various groups varied less than 3 gm. in any series of tests.

Furthermore, when small numbers of rats are used in each group, it is imperative that litter mates be distributed throughout the various groups. In the present work there were three litters where the rats consistently gained far under the average on the various diets. When a small number of animals is used, variations might be noted especially if two from such a litter were assigned to one group. It should also be noted that in the experiments of Schantz et al. ('40), only carotene was used as a supplement for vitamin A in the tests with the vegetable oils while obviously the animals on butter diet received also vitamin A. In the present tests, both the provitamin A and vitamin A have been supplied in all diets.

Boutwell et al. ('43) have extracted the skimmed milk powder extensively in their most recent tests while we have used unextracted milk powder where the lipids amounted to 1.10%. However, in the earliest tests of Schantz et al. ('40) where liquid skimmed milk was used, no such extraction was employed; it was in these experiments where the most marked variations in growth occurred at 3 weeks.

The reason for the poor results of Boer ('41) with olive oil might in all probability be ascribed to the strong flavor. These differences were not observed in our studies. The olive oil used by us was the poorest of the fats, judged by the relatively high free fatty acid content. Also Clausen, Barnes and Burr ('43) have shown that the growth on diets containing various fats is markedly influenced by rancidity; they have indicated that "The inadvertent destruction of dietary essentials and the possible independent toxicity of rancid fat are factors that might confuse the interpretation of many diet experiments".

SUMMARY

No differences in the growth of weanling rats were noted at any time over a 12-week period when they were fed mineralized skimmed milk powder, vitamin supplements and butter as compared with corn, cottonseed, olive, peanut or soybean

oils or margarine. The extent of growth was confirmed at 3 and 6 weeks by x-ray determinations of tibia length. Also, the efficiencies of conversion of these various fats to body tissue were identical. These experiments refute the idea that butter fat possesses certain saturated fatty acids, not present in other fats, which are essential for growth.

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NUTRITIONAL DERMATOSES IN THE RAT

IX. EVALUATION OF THE INTERRELATIONSHIP OF MAGNESIUM DEFICIENCY AND DEFICIENCIES OF THE VITAMIN B COMPLEX¹

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ONE PLATE (ELEVEN FIGURES)

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Deficiency of the vitamin B complex has been considered a factor which influences the signs of magnesium deficiency (Watchorn and McCance, '37; Greenberg and Tufts, '38). In this communication the signs of "uncomplicated" magnesium deficiency are described and the question of the interrelationship of the deficiencies of the vitamin B complex and magnesium deficiency is evaluated.

EXPERIMENT I

"UNCOMPLICATED" MAGNESIUM DEFICIENCY

For the production of "uncomplicated" magnesium deficiency the following diet was employed: vitamin-free² casein, 200 gm.; dextrin, 541 gm.; butter fat, 100 gm.; McCollum salts no. 62 (Kruse, Orent and McCollum, '32) 59 gm. Crystalline supplements of 20 mg. of thiamine hydrochloride, 10 mg. of riboflavin, 10 mg. of pyridoxine, 30 mg. of nicotinic acid, 15 mg. of calcium pantothenate, 1 gm. of choline hydrochloride, 375 mg. of inositol, and 375 mg. of para-amino-benzoic acid were incorporated into the diet. Thiamine hydrochloride, riboflavin, calcium pantothenate, pyridoxine, and nicotinic acid were calculated on the basis of the amounts of these vitamin B complex factors which are contained in 100 gm. of yeast³ of known assay (Sullivan and Evans, '43). Five hundred milligrams of cystine and 18,200 I. U. of vitamin A, and 5,460 I. U. of vitamin D⁴ were included in the ration.

¹ Aided by a grant from the Rockefeller Foundation Fluid Research Fund.

² S. M. A. Corporation.

³ Anheuser-Busch.

⁴ Mead Johnson's Oleum Percomorphum.

Each animal received supplements of 10 mg. of alpha-tocopherol per week. The diet was planned to include all of the known essential elements and was considered to be otherwise adequate except for magnesium, of which it was calculated to contain less than 1.8 mg. in each kilogram. The control diet differed from the deficient diet in that 1 gm. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was triturated into the salt mixture and was added at the expense of 1 gm. of dextrin to each kilogram of the diet.

Two or 3 days after the experimental ration was fed to recently-weaned rats the animals were nervous and hyperirritable. Convulsive seizures occurred spontaneously and were precipitated by noise or handling any time after the first week. The average duration of the convulsive seizures was 2 minutes; it varied from 1 to 14 minutes. Some of the animals succumbed immediately after the convulsions; others survived to experience several subsequent seizures. In twenty-nine litters consisting of 200 animals, eleven were observed at various times to have experienced at least three convulsive seizures and it is likely that at unobserved times, especially at night, additional seizures occurred. The tonic-clonic convulsive seizure has been described by several investigators since it was first reported by McCollum and Orent ('31). The animal races wildly around the cage in great confusion. It falls on its side and is extremely spastic in the first stage of the seizure; the eyes are closed tightly; the extremities are hyper-extended stiffly for several seconds, after which the eyelids gradually open, the animal relaxes, and the extremities are flexed rapidly. This stage is usually followed by a stage in which the animal is again rigid and opisthotonic; the animal then lies in a semi-somnolent state; it is listless, flaccid, and clumsy for a few minutes before it recovers or dies.

The first cutaneous sign was erythema; it occurred on the fourth to the eighth day, depending upon the litter. As a rule, the first sites involved by erythema were the trunk and/or the conchae of the ears. Again this depended upon the litter as the implicated sites were uniform for each group of animals. Erythema spread rapidly and on the trunk it was generalized in some animals and/or distributed in numerous non-grouped multiform plaques in others. From the conchae the erythema spread peripherally to the tips of the ears and it was usually of an intensity that suggested hemorrhage (fig. B). Shortly afterwards the areas involved by the erythematous plaques and the diffuse erythema were edematous. In the case of the ears this was striking as they usually were swollen to approximately twice their normal thickness (fig. B). Coincident with or soon after the initial cutaneous signs the paws were swollen and red. The edema and erythema

of the paws were not preceded or accompanied or followed by scaling. During the erythematous stage the animals continued to manifest signs of irritability. Convulsive seizures occurred frequently but not necessarily in the erythematous stage. The initial stage of erythema lasted as a rule from 4 to 7 days, but in some litters the stage was shorter; it was followed by a period during which there was pallor. Hyper-irritability continued for some time after the erythema subsided. There was no change in the lustre of the fur and alopecia was not observed in the early stage.

Growth during the first 2 or 3 weeks approximated that of rats in the control groups. Thereafter there was less rapid rate of growth. In animals that survived for 3 months the increment of weight was 100% and the peak of the weight curve was reached between the eighth and the tenth week. Thereafter until death there was a slight decrease in weight.

As the disease progressed the animals were weak and less irritable. In the late stage the animals were listless and reluctant to be handled; their behavior was of a sullen type. Priapism occurred in the majority of male animals. Although spontaneous convulsions were infrequent in the late stage, they occasionally occurred and were induced a few times. In three litters there was widespread, diffuse alopecia of the venter. Kruse, Orent and McCollum ('32) observed alopecia in the late stage of the deficiency. It has been emphasized before that the signs and symptoms in a late stage of a deficiency are unimportant insofar as they aid in evaluating specific effects of a deficiency. This is especially true in the magnesium-deficient rat because after the sixth to the eighth week there is nutritive failure and there are extensive internal changes (nephritis, hepatitis) which vary in different rats and which predispose to superimposed illness, as well as contribute to the general state and symptoms of the sick animals. Occasionally there was hair loss which was confined to individual plaques of eczematous dermatitis that occurred at the sites previously involved in the early stage by the erythematous, edematous plaques. In some cases there was a crusting and scaling of the margins of the ears (fig. C) and in many animals the ears remained thickened until death. In no instance was there distortion of the contour of the ears. Scattered plaques of dermatitis involved every portion of the skin of some animals. In the tails of a few there were extensive changes which included dermatitis, induration, ulceration and gangrene. Frequently after 8 weeks there were ulcers on the plantae. A characteristic sign of the deficiency was the manner in which the skin of the tail was detached

from the fibrous tissue immediately surrounding the bone of the tail. When an animal was suspended by the tail, the skin slipped from its attachment to the deeper structure. By gently rotating the tail between the thumb and forefinger, this phenomenon of slippage was readily demonstrated. During the late stage the skin was thickened and tougher; when it was cut with a knife it offered more resistance than the skin of control animals. The skin lost its resiliency. If the skin of the back was grasped between the thumb and the forefinger and lifted into folds it failed to "snap back" rapidly into place after releasing the thumb and forefinger. Edema of the dependent parts persisted until death.

Microscopic findings

During the first week of the deficiency there were no epidermal changes. The initial cutaneous change consisted of edema in the lower half of the cutis; the blood vessels in the edematous region were dilated and hyperemic, and there was mild, diffuse cellular infiltration throughout the cutis. In the second week there was a slight amount of loosely lamellated hyperkeratosis. Intraepidermal vesiculation was the first evidence of epidermal change. In the cutis there was cellular infiltration. During some stages the predominating cell was a mononuclear one with non-granular cytoplasm that stained diffusely with eosin. There was hyperemia in the upper as well as the lower portion of the cutis (fig. G). In some sections the edema persisted, while in other sections it subsided somewhat. In the areas of edema there was dissociation of connective tissue. In some regions there were patchy acanthosis and an increase in intraepidermal vesiculation. The edema of the cutis persisted in many sections. The extent of the cellular infiltration increased in the upper cutis, and exudative cells encroached upon the epidermis. In some sections there was a perifollicular localization of the cellular infiltration. Hyperemia was of variable extent. In the period from the fourth to the sixth week there were more epidermal changes; many of the individual cells in the several layers were vacuolated and the nuclei were shrunken. There was extrusion of keratohyalin granules into the stratum corneum and in some sections there were areas of parakeratosis and crusting. In a few areas there were small ulcers. During the period from the seventh to the fifteenth week, depending upon the area of skin examined, there was variation in the extent of cutaneous alteration, ranging from a barely discernible amount of intraepidermal edema to extensive vesiculation, with and

without crusting, to ulceration. Small and large sections of acanthotic epidermis were punctuated by areas of apparently normal epidermis. Hypermia and edema persisted in many of the sections of the paws. However, there was subsidence of the edema in many other areas, and the cellular infiltration was less dense, except in the cutis beneath those portions of the epidermis in which there was vesiculation. After the fifteenth to the twentieth week the epidermis was of normal thickness, except in acanthotic and eczematous portions. However, the thickness of the cutis often was increased considerably. There were no calcified plaques or sclerodermatous changes. Van Kossa stains revealed a small amount of calcium in the epidermis but none in the cutis, and elastic tissue stains demonstrated no decrease in, or disturbance of, elastic tissue. Throughout the disease the sebaceous glands and rudimentary coil glands were not affected except in ulcerated areas.

In the kidneys extensive degeneration of the tubules and the glomeruli and deposition of calcareous material in the areas of degeneration were observed. In the livers of many of the animals there were hyperemia, perivascular edema and occasionally disintegration of the liver cells.

Chemical findings

Moisture. Watchorn and McCance ('37) studied the moisture content of the skins of 90-day-old rats suffering with chronic magnesium deficiency. They reported that the moisture content of the skin of the deficient rats was 57.9% compared to 55.8% in normal rats. Fawns and Jung ('33) found that the moisture content of normal rat skin varied between 42.7 and 56.8%. It was considered desirable to ascertain the percentage of moisture in acute magnesium deficiency, with which we were dealing in this study, and to determine whether the alternating stages of erythema and pallor influenced the amounts of moisture. The entire body was closely shaved with small animal clippers. The skin was immediately excised, stripped of loose subcutanea and promptly weighed. The tissue was then dried for 48 hours at 105°-110° in order to obtain a constant weight. The moisture of the skins of the deficient rats in this experiment (table 1) varied from 29.1 to 41.7%; in the control group the moisture content varied from 25.13% to 47.9%. There were no significant differences in the two stages, namely erythema and pallor, of acute magnesium deficiency. The increase in moisture reported by Watchorn and McCance was probably a manifestation of the chronic stage of the deficiency and the difference between the percentage of moisture content in the rats in this ex-

TABLE 1
Showing the moisture and ash, magnesium and calcium contents of the skins of magnesium-deficient rats reared on experimental rations supplemented with adequate and inadequate amounts of the vitamin B complex.

NUMBER OF RAT	WEIGHT IN GRAMS	AGE IN DAYS	MOISTURE		ASH ²		MAGNESIUM ³		CALCIUM ⁴		CA. MG. RATIO
			Deficient	Control	Deficient	Control	Deficient	Control	Deficient	Control	
			%	%	%	%	mg./100 gm.	mg./100 gm.	mg./100 gm.		
M1	68	35	34.9		52.0		20.38		67.99		3.3
N1	75	35		38.7		61.9		21.33		42.28	1.9
M2	69	35	34.0		50.1		16.33		20.23		1.2
N2	79	35		34.5		51.1		18.59		26.72	1.4
M3	62	35	32.1		45.9		17.45		18.10		1.1
N3	103	35		43.2		74.8		23.80		20.28	0.85
M4	83	35	34.3		50.8		18.78		12.67		0.67
N4	92	35		47.9		71.9		21.40		14.08	0.66
M5	85	49	33.9		52.4		12.90		13.68		1.1
N5	87	49		26.7		50.9		16.70		16.92	1.0
M6	118	49	41.7		70.5		23.70		15.31		0.65
N6	91	49		32.9		47.8		15.49	
M7	78	49	29.1		39.8		14.52		12.43		0.85
N7	78	49		26.3		35.4		16.74		12.18	0.73
M8	60	49	33.8		40.8		18.45		12.00		0.65
N8	85	49		47.4		60.2		17.57		8.77	0.50
X1	76	35	45.5		40.3		22.45		20.88		0.93
Y1	67	35		31.7		46.1		20.80		17.84	0.86
X2	83	35	32.0		59.2		15.81		13.96		0.88
Y2	67	35		35.7		54.3		20.33		17.59	0.87
X3	61	35	37.7		45.6		21.39		22.31		1.0
Y3	79	35		41.3		69.4		17.46		4.62	0.27
X4	72	35	34.3		50.7		13.46		14.82		1.1
Y4	84	35		29.1		39.8		16.79		8.99	0.54
X5	86	49	37.4		58.6		12.97		15.70		1.2
Y6	74	49		42.8		73.8		17.89		16.71	0.93
X6	90	49	33.3		48.7		13.62		11.74		0.86
Y6	83	49		38.9		64.2		16.14		16.18	1.0
X7	92	49	33.3		48.9		12.53		14.44		1.2
Y7	57	49		31.8		45.6		16.67		19.72	1.2
X8	81	49	31.3		44.5		12.12		9.82		0.81
Y8	75	49		34.6		51.1		18.95		12.32	0.65

¹ Calculated on a dry basis.

² Methods of Analyses of the Association of Official and Agricultural Chemists, 4th Ed. 1935, p. 122.

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⁴ Pike, S. H., and Y. Subbarow 1925 The colorimetric determination of phosphorus. J. Biol. Chem., vol. 66, p. 375.

⁵ Greenberg, D. M., and C. E. Larson 1938 The analysis of calcium in blood and other biological material by titration with ceric sulfate. J. Biol. Chem., vol. 123, p. 191.

periment and those of Fawns and Jung ('33) and Watchorn and McCance ('37) may be due to the fact that the fat stores of the skin in the acute stage were not depleted.

Ash content. Two litters of 4-week-old rats were carefully matched and divided into groups M and N (table 1). One week later half of each group was sacrificed for analyses and 3 weeks later the other half was sacrificed for analyses (table 1). The results showed that the ash content of the deficient rats (M 1, 2, 3 and 4) varied from 45.9 to 52%; the ash content of the control rats (N 1, 2, 3 and 4) varied from 51.1% to 74.8%. This apparent difference in the ash content of groups M and N is not due to the slight differences in the magnesium content.

Magnesium content of the skin. The magnesium content of normal rat skin has been determined by Watchorn and McCance ('37). In normal rats, 90 to 120 days old, they found 29.6 mg. per 100 gm., as calculated on the basis of dry weight, and in magnesium-deficient rats of the same age 24.9 mg. Tufts and Greenberg ('38) reported that the magnesium content of the skins varied from 9.9 to 21.8 (mean 15.1) mg. per 100 gm. of fresh tissue. Their figures are not comparable to those in table 1, inasmuch as the values in this experiment are expressed in milligrams per 100 gm. of dry tissue. In their control group Tufts and Greenberg found that the magnesium content of the skin varied from 10 to 20.3 (mean 15.1) mg. per 100 gm. of fresh tissue.

In table 1 are summarized the magnesium and calcium values for magnesium-deficient rats 1 and 3 weeks after the institution of the "uncomplicated" magnesium-deficient diet. These figures represent the values for the acute stage of the disease when the rats were 35 and 49 days old. The values reported by Watchorn and McCance represent findings in the later stage. In the normal group (N 1, 2, 3, 4, 5, 6, 7 and 8) the magnesium content varied from 18.59 to 23.80 mg. per 100 gm. at 35 days, and from 15.49 to 17.57 mg. per 100 gm. at 49 days. In the deficient group (M 1, 2, 3, 4, 5, 6, 7 and 8) the magnesium content varied from 16.33 to 20.38 mg. per 100 gm. at 35 days (stage of erythema) and from 12.90 to 23.70 mg. per 100 gm. at 49 days (stage of pallor). These findings showed that the magnesium content of the skin of normal and deficient rats was somewhat lower at 35 and 49 days of age than at 90 to 120 days of age (Watchorn and McCance, '37). However, there was slight, if any, significant reduction of the magnesium content in the magnesium-deficient animals as compared with their litter-mate controls. There was no significant difference in the stages of erythema and pallor.

Calcium content of the skin. The calcium values showed no consistent rises in the stage of erythema. However, the highest values for calcium were during this stage. The calcium-magnesium ratios failed to furnish the anticipated information that calcium values in the skin would be increased simultaneously with a decrease in magnesium.

Differential diagnosis

The dermatoses resulting from deficiencies of the entire vitamin B complex other than crystalline thiamine (Sullivan and Nicholls, '40b), riboflavin (Sullivan and Nicholls, '41), pantothenic acid (Sullivan and Nicholls, '42b) and biotin (Sullivan and Nicholls, '42a) bear no gross or microscopic resemblances to the signs produced by deficiency of magnesium. Pyridoxine deficiency (Antopol and Unna, '39; Sullivan and Nicholls, '40a) is the only nutritional dermatoses that need be differentiated, because the involvement of the peripherae and the reported convulsive seizures (Chick, El Sadr, and Worden, '40) in pyridoxine deficiency may be sources of confusion during certain stages of the diseases. There is also some similarity in the weight curves of pyridoxine-deficient rats and magnesium-deficient rats. However, there are gross and microscopic features which distinguish the two diseases. In pyridoxine deficiency (fig. K) desquamation of the skin of the peripherae was characteristic while in magnesium deficiency (fig. J) erythema and edema of the paws were not accompanied, preceded, or followed by scaling. In pyridoxine deficiency the normal contour of the ear was distorted; the ears were shrivelled and shrunken (figs. D, E and H). In magnesium deficiency the ears were thickened and crusts and scales formed on the margins but the contour was not destroyed (figs. B, C and G). Microscopically there are distinguishing features in the cutaneous alterations. In pyridoxine deficiency there was hyperkeratosis and a peculiar type of acanthosis (fig. H). In magnesium deficiency (fig. G) there were extensive edema and hyperemia in the lower cutis, a diffuse cellular infiltration throughout the cutis and an increase in the thickness of the cutis. Chick, El Sadr, and Worden ('40) reported convulsive seizures in rats maintained for long periods, 4 to 5 months and over, on a purified synthetic diet deficient in pyridoxine. The seizures were similar in many respects to those observed in the magnesium-deficient rats, except that there was flaccidity rather than rigidity in the initial stage of the seizures of the pyridoxine-deficient rats. Also the long period of the experiment, 4 to 5 months, which preceded the onset of the convulsions in the rats of Chick and asso-

ciates was at variance with the short period, less than a week, which preceded the onset of convulsions in the magnesium-deficient rats. The extensive kidney changes and edema of the dependent parts which were characteristic of magnesium deficiency were never observed in pyridoxine deficiency.

EXPERIMENT II

THE LACK OF INFLUENCE OF THE VITAMIN B COMPLEX ON THE SIGNS OF MAGNESIUM DEFICIENCY

Greenberg and Tufts ('38) presented evidence purporting to show that the magnesium deficiency syndrome produced in rats by Kruse, Orent and McCollum ('32) was complicated by vitamin B complex deficiency. Watchorn and McCance ('37) stated that some of their magnesium-deficient animals showed signs of vitamin B complex deficiency although the controls were normal. This suggested that the requirements of the vitamin B complex were higher or that vitamins of the B complex functioned less efficiently when the magnesium level was low. In order to examine the possible relationship of the vitamin B complex and magnesium deficiency, the signs and symptoms produced by several variations of the magnesium-deficient diet were evaluated.

A. A comparison of magnesium deficiency signs produced by diets containing crystalline vitamin B complex supplements of high and low levels

The signs of magnesium deficiency were studied in two groups of carefully paired young litter mates. To one group was fed the diet and supplement described in experiment I; the amount of vitamin B complex therefore corresponded to a 10% level of a potent yeast. To the other group was fed the same diet with the exception that the crystalline B vitamins were incorporated at a level that corresponded to the amount of the B vitamin in 1% yeast. The onset and severity of the cutaneous signs of magnesium deficiency were essentially the same for each group. Growth was inferior in the latter group. The convulsions were not delayed in the latter group but occurred at the same period (within 2 days) of the convulsions in the group that was adequately supplemented with the vitamin B complex. In three of the litters of the first group there developed in the late stage certain non-specific, cutaneous signs which included mild, generalized, brown scaling on the back, dishevelled fur and alopecia on the venter. These signs vaguely suggested superimposed riboflavin deficiency. Adminis-

tration of large doses of riboflavin and the entire vitamin B complex supplement had no effect on the signs.

Chemical findings

Sixteen 28-day-old rats, that were the litter mates of the rats in groups M and N of experiment I, were evenly matched and divided into two additional groups, X and Y (table 1). To group X was fed a magnesium-deficient diet containing crystalline vitamin B complex supplements, incorporated at a level corresponding to 1% yeast. To group Y was fed a magnesium control diet also containing crystalline vitamin B complex supplements, incorporated at a level corresponding to 1% yeast. The moisture content, the percentage of ash, the amount of magnesium and calcium per 100 gm. of dry tissue, and the calcium-magnesium ratios of the skin of half of each group were determined 1 week later during the stage of erythema and 3 weeks later during the stage of pallor. These results were compared with the values for groups M and N, that were supplemented with an adequate amount of vitamin B complex. The data indicated that the level of the vitamin B complex was unimportant insofar as these values were concerned. In the vitamin B complex-deficient magnesium-deficient rats the magnesium content at 35 days of age was approximately equal to that of the control groups (N and Y) that were supplemented with adequate and inadequate vitamin B complex. However, at 49 days the magnesium content of the skins of the vitamin B complex, magnesium-deficient rats was slightly lower than that of their controls and of groups M and N.

B. A comparison of the therapeutic response of vitamin B complex deficiency other than thiamine hydrochloride to treatment with magnesium-deficient diets containing high and low levels of the vitamin B complex

Three groups of young animals were fed a diet deficient in the entire vitamin B complex except crystalline thiamine hydrochloride. The animals lost weight. One month later when they were practically in extremis, and therefore could be considered to have been well depleted of the vitamin B complex other than thiamine, the original ration was discontinued. Microscopic examination showed atrophy of the epidermis, the sebaceous glands and the cutis. The rats were then divided into two groups to which magnesium-deficient diets containing inadequate and adequate amounts of the vitamin B complex were fed.

To one group, the vitamin B complex was supplied as crystalline factors incorporated at a level equivalent to 1% yeast of known potency. To the other group the vitamin B complex was incorporated as crystalline factors in amounts equivalent to the vitamin B complex contained in 10% yeast. All of the animals recovered from their almost moribund state. The rats in the group that received the vitamin B complex, equivalent to 1% yeast, gained 6 gm. during the first month after the magnesium-deficient diet was started; the rats in the group that received the vitamin B complex equivalent to 10% yeast gained 29 gm. during the month after the magnesium-deficient diet was started. The onset of hyperemia and hyper-irritability and the convulsive seizures occurred within 3 to 4 days in the two groups, and the duration and severity of the cutaneous signs were identical. On microscopic examination it was found that the epidermis and cutis were no longer atrophic and that there was partial to complete restitution of the previously atrophic sebaceous glands; in the lower cutis there were edema and hyperemia and throughout the cutis there was a cellular infiltration characteristic of magnesium deficiency. These findings demonstrated that the addition of adequate and inadequate amounts of the crystalline supplements of the vitamin B complex resulted in a cure of the signs due to deficiency of the vitamin B complex other than thiamine, in skin manifesting histologic evidence of magnesium deficiency.

C. The evaluation of magnesium deficiency signs in young rats reared on a diet containing a commercial liver extract known to be low in magnesium and pyridoxine

The signs of magnesium deficiency were evaluated in ten young rats reared on a magnesium-deficient diet in which a commercial liver extract⁵ known to be low in magnesium was incorporated at a 1.5% level as the source of the vitamin B complex. The levels of the individual components of the vitamin B complex were considerably less than those in experiment I. The time of the onset and duration of the signs corresponded to those of experiment I. In another litter of rats the amount of the commercial liver extract was doubled and the onset, duration and severity of the signs were not influenced. The amount of pyridoxine in this source of the vitamin B complex is com-

⁵ Lederle's Vitamin B Complex. Each ml. contains approximately 375 μ g. of thiamine hydrochloride, 150 μ g. of riboflavin and 50 μ g. of pyridoxine. When incorporated at a 1.5% level the amount of pyridoxine was approximately one-twelfth that which the rats in experiment I received. When the amount of the extract was doubled the pyridoxine content was still comparatively low.

paratively low; therefore additional daily supplements of 500 μ g. of crystalline pyridoxine were administered to two rats. The additional pyridoxine had no influence on the signs.

D. The lack of influence of massive doses of pyridoxine on the signs of magnesium deficiency

To two litters of 21-day-old rats the diet described in experiment I was fed. The supplements of pyridoxine were increased five-fold, i. e., from 10 mg. to 50 mg. Again there was no influence on the cutaneous signs, the irritability and the convulsive seizures.

E. Lack of influence of massive doses of thiamine, riboflavin, pyridoxine, pantothenic acid, para-amino-benzoic acid, nicotinic acid and choline on the signs of magnesium deficiency

In order to complete the observations on the possible influence of the vitamin B complex on the signs of magnesium deficiency, the crystalline vitamin B complex supplements listed above were incorporated into the diet at a level five times as great as that described in experiment I. This had no influence on the onset or severity of the cutaneous signs, the hyperirritability and the convulsive seizures.

EXPERIMENT III

LACK OF THE INFLUENCE OF VITAMIN E ON MAGNESIUM DEFICIENCY SIGNS

The supplement of alpha-tocopherol used throughout these experiments was omitted from the rations of five rats in a litter of eight. There was no influence on the onset, duration and severity of the signs.

SUMMARY AND DISCUSSION

The signs resulting from magnesium deprivation were described in 1931-32 by McCollum and Orent, and Kruse, Orent and McCollum. These investigators produced a symptom-complex comprising vasodilatation, hyperirritability of the nervous system, cardiac arrhythmia, spasticity and tonic-clonic convulsions. The experimental diet included only 1.8 parts per million of magnesium, but was considered otherwise adequate. A yeast extract corresponding to approximately a 13.5% level was incorporated into the ration. Assuming that the yeast was a potent source of the vitamin B complex, the diet was probably adequate in all respects, except that it lacked magnesium and vitamin E. Inas-

much as the signs occurred within 1 to 3 weeks, it is likely that the inclusion of vitamin E would not have affected the signs. In 1938, Greenberg and Tufts studied the influence of several factors on the incidence, time of onset, and duration of the peripheral vasodilatation and hyperirritability in magnesium-deficient rats. They stated that the signs were affected by the level of the vitamin B complex, and criticized the use of the alcoholic extract of yeast employed by Kruse, Orent and McCollum, because it was apparently low in factors of the vitamin B complex. Of the several deficiency syndromes produced by deficiencies of the components of the vitamin B complex, pyridoxine deficiency is the only one likely to be confused with magnesium deficiency, because of the apparent similarity of the involvement of the peripherae and the reported convulsive seizures in pyridoxine deficiency (Chick, El Sadr and Worden, '40). The findings in experiment I demonstrated that: (a) the gross signs and symptoms of magnesium deficiency may be differentiated readily from those of pyridoxine deficiency; and (b) the histologic findings in the two diseases are dissimilar. The findings in experiment II demonstrated that: (a) the level of the vitamin B complex exerted no influence on the onset and/or severity of the cutaneous signs and the convulsive seizures, and (b) magnesium deficiency signs in the skin could be produced simultaneously with the healing of the signs of deficiency of the vitamin B complex. The data in experiment II also showed that the magnesium content of the skin is similar in rats subsisting on diets containing adequate and inadequate vitamin B complex.

In acute magnesium deficiency the percentage of magnesium of the skin was found to be only slightly lower than the percentage in normal animals. Likewise, the moisture and ash contents and the level of calcium were not significantly altered. When the chemical findings of the magnesium-deficient, vitamin B complex-deficient rats were compared with those of the magnesium-deficient rats that were adequately supplied with the vitamin B complex, the data showed that there was no appreciable influence of the vitamin B complex on the chemical findings.

In experiment III it was demonstrated that vitamin E was unrelated to the signs of magnesium deficiency.

CONCLUSION

The data in these experiments show that there is no significant interrelationship of magnesium deficiency and the deficiencies of the vitamin B complex.

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PLATE

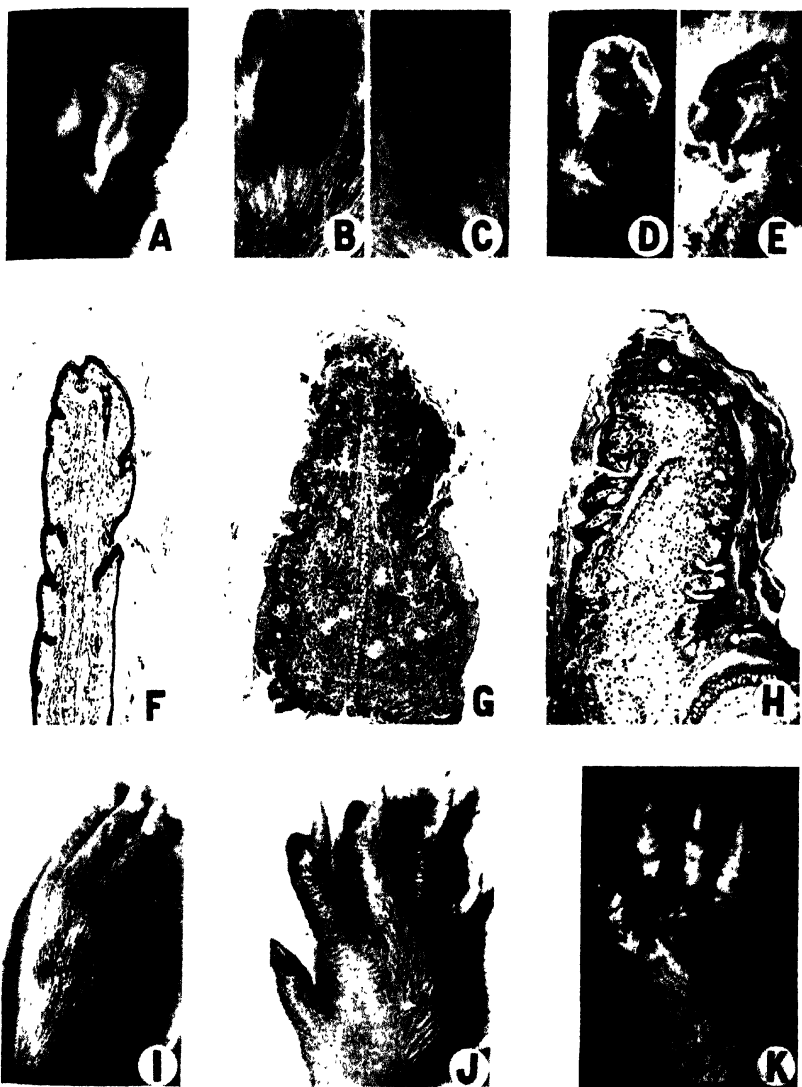
PLATE 1

EXPLANATION OF FIGURES

In the left-hand column at A, F and I are portrayed a normal ear and a normal foot. In the middle column are portrayed at B, C, G and J signs of magnesium deficiency. In the right-hand column at D, E, H and K are signs of pyridoxine deficiency.

In the early stage of magnesium deficiency (B) the ear is erythematous and swollen; the contour of the ear is not disturbed. In the late stage of magnesium deficiency (C) the ears are thickened and on the margins there is crusting and scaling. In the early stage of pyridoxine deficiency (E) the ear is shrivelled and shrunken. Microscopic changes of the ear in magnesium deficiency (G) consist of edema, diffuse cellular infiltration throughout the cutis, hyperemia, acanthosis alternating with apparently normal epidermis, and slight if any hyperkeratosis. On the tip of the ear there is ulceration and crust formation. In pyridoxine deficiency the cartilage is distorted, there is only a slight amount of cellular infiltration in the cutis, the acanthosis is of a different type and there is extensive hyperkeratosis.

In magnesium deficiency the paws (J) are swollen and erythematous. Desquamation does not precede, accompany or follow the erythematous stage. In pyridoxine deficiency (K) scaling of the dorsa of the paws is a characteristic feature.



NUTRITION AND TOLERANCE TO ATABRINE¹

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Little study has been made of the effect of continuous drug administration upon the nutritive requirements, or conversely, of the effect of nutritive state upon the ability of the animal to withstand pharmacological agents. However, a survey of the literature reveals sufficient evidence to indicate that the action of some drugs may depend to a certain extent upon the diet of the animal. The ability to raise animals as the rat, chick, and dog, on diets of more or less known constitution affords a tool for the investigation of the relation between nutrition and pharmacology.

Perhaps the best known example of such a relationship is that of certain sulfonamides. Black, McKibbin and Elvehjem ('41) showed that on a purified diet supplemented with the crystalline vitamins the inclusion of sulfaguanidine in the diet depressed the rate of growth of young rats. This effect could be prevented by feeding liver extract and partially overcome by para-aminobenzoic acid. Similar and more extensive work of various laboratories has been reviewed (Anonymous, '43a, '43b). Deficiencies of biotin, folic acid and vitamin K have been reported in rats receiving sulfaguanidine or succinylsulfathiazole. It seems apparent that these nutrients, on the experimental diets used, were supplied by an intestinal flora which became inadequate in the presence of the sulfonamides. Kornberg, Daft and Sebrell ('43) have shown that the granulocytopenia and anemia produced in rats by feeding sulfonamides can be cured by liver extracts. Xanthopterin (Totter and Day, '43) may be one of the factors involved. Daft and coworkers (Daft, et al., '43) have presented evidence that muscle lesions caused by feeding sulfasuxidine are actually an alpha-tocopherol deficiency.

Less evidence is available on the effect of diet upon the therapeutic action of the sulfonamides. However, McCarty ('41), Selbie ('40),

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

Maier and Riley ('42), Findlay ('40) and others have shown that p-aminobenzoic acid will abolish the therapeutic action of these agents in several diseases.

The toxicity of several other agents has also been related to diet. The carcinogenicity of butter yellow apparently depends upon protein (Kensler et al., '41) or choline and cystine (Gyorgy et al., '41) and biotin (du Vigneaud et al., '42). Methionine and cystine prevent growth inhibition of butter yellow on low protein diets (White, '40). High protein diets also decrease the toxicity of trinitrotoluene (Himsworth and Glynn, '42) and of selenium (Smith and Stahlman, '40; Anderson et al., '41).

The detoxification of certain arsenicals by para-aminobenzoic acid has been demonstrated by Sandground and Hamilton ('43a, '43b). It may be important from the therapeutic standpoint that the para-aminobenzoic acid did not inhibit the trypanocidal action of the arsenicals, although their toxicity was much decreased.

The antimalarial drugs, atabrine and quinine, are administered prophylactically and in suppressive treatment over long periods of time. Because malaria is endemic in so many regions and particularly because certain currently strategic areas are highly infested with malaria, any knowledge relating diet to tolerance of antimalarials is opportune. This paper reports experiments designed to determine whether various dietary regimens affect the toxicity of atabrine over extended periods.

EXPERIMENTAL*

All of the studies on rats were made with young albino rats weighing 40 to 60 gm. purchased from the Rockland Farms. They were housed in individual cages, and groups of 5 or 6 rats were fed each experimental ration ad libitum except where otherwise noted.

The experimental rations used in most of the studies were as follows: sucrose 74%; casein³ 18%; salt mixture 4% (Hegsted et al., '41); and corn oil 4%. Vitamins were added at the following levels per 100 gm. of ration: thiamine chloride 100 µg., riboflavin 200 µg., pyridoxine 100 µg., calcium pantothenate 1.6 mg., nicotinic acid 1.0 mg., and choline 150 mg. Vitamins A, D and E were fed by dropper twice weekly to supply 80 I. U. of A, 1.5 I. U. of D, and 0.5 mg. of alpha-

* We are indebted to Merck & Company, Rahway, New Jersey, for furnishing the crystalline B-vitamins and the alpha-tocopherol, and to Abbott Laboratories, North Chicago, Illinois, for furnishing Haliver Oil.

³ S. M. A.

tocopherol per rat per week. The above levels of the water soluble vitamins were chosen for the original experiments to be near or slightly below the optimum level since it was thought that if the requirement of any of these was increased by atabrine it would be detected at these levels. In later experiments, when a single vitamin was investigated, it was simply omitted from the diet and the levels of the other vitamins doubled, except choline, which was fed at 100 mg. per 100 gm. of ration. Atabrine dihydrochloride was added to the appropriate diets by dissolving in alcohol and drying on the ration.

The general plan for the study of the vitamins which were investigated was to deplete two large groups of animals, one of which received atabrine (usually 40 mg. per 100 gm. of ration) in the diet. When the animals were depleted (symptoms or failure to gain), they were divided into groups and fed graded levels of the vitamin in supplement cups or by dropper. The gains of the animals with and without atabrine and receiving the same level of supplement were compared.

In studies with chicks, day-old White Leghorn cockerels were used; they were housed in groups of six in heated cages and fed the appropriate ration and water ad libitum.

RESULTS

General

Atabrine has been fed to rats at levels from 0 to 65 mg. per 100 gm. of ration. Food consumption records of these young animals showed that they consumed an amount equal to approximately 10% of their body weight per day. Thus atabrine levels per 100 gm. of ration in short experiments are similar to levels in terms of per kilogram of body weight per day. Over a 6 months' period atabrine levels of 25 mg. % or less caused no growth retardation or other abnormalities. Over a 3 months' period levels of 40 and 65 mg. % prevented normal growth (20 to 35% less) but except for yellowing of the skin and a ruffled coat, no other abnormalities were noted. Actual atabrine intake of these animals is unknown but is somewhat less than suggested, as rats of this age consume less food relative to body weight.

Rather complete histological studies were made of a few rats which had received atabrine at levels of 25 and 40 mg. % for 6 months. Only minimum micropathologic changes were noted. These consisted of the appearance of granules in the Kupffer cells of the liver, the glomerular epithelium and occasionally the tubular epithelium of the kidney, in the spleen and in heart muscle. These granules are presumably ata-

brine. In teased-out preparations of kidney placed in strong potassium iodide solution the granules are very conspicuous and decidedly brownish in color. The early storage of atabrine or an atabrine derivative in the epithelial cells covering the glomerular capillaries is of interest. No necrotic changes were detected.⁴

A diet containing all of the vitamins at five times the levels given above and another containing brewers' yeast at a level of 4% were fed with and without 40 mg. % of atabrine. Although the growth was somewhat improved, the atabrine groups still gained at approximately 25% less than the control. Apparently growth inhibition is not due to lack of intestinal synthesis of nutrients furnished by yeast nor can it be overcome by high levels of the known vitamins.

TABLE 1

Average gain in weight on various levels of riboflavin with and without atabrine in the diet.

MICROGRAMS OF RIBOFLAVIN PER DAY	TIME OF EXPERIMENT	NO ATABRINE	PLUS ATABRINE	PLUS ATABRINE AS % OF CONTROLS
Experiment I	<i>days</i>	<i>grams gain</i>	<i>grams gain</i>	
0	25	11.5	11.5	100
6	25	48.0	35.5	74
12	25	...	40.5	...
30	25	...	55.5	...
Experiment II				
4	43	55.5	42.6	77
8	43	80.0	71.8	89
12	43	99.0	81.5	82

Riboflavin deficiency

Thirty-six male rats were depleted of riboflavin, twenty-four of which were on the diet containing 40 mg. % of atabrine. After 57 days the controls were divided into two groups which received 0 and 6 μ g. of riboflavin per day, and the atabrine rats into four groups which received 0, 6, 12, and 30 μ g. of riboflavin per day. A comparison of the two groups receiving 6 μ g. of riboflavin (table 1) shows that, as on a complete diet, the atabrine rats gained about 25% less than the controls. Over 12 μ g., but less than 30 μ g., of riboflavin per day were required before the atabrine groups equaled the gain of the controls. No difference was noted in the two groups which received no riboflavin.

⁴The pathologic examinations, including preparation of the sections, were done by Dr. S. B. Wolbach, Harvard Medical School, for which the authors are deeply grateful.

In a confirmatory experiment the depletion period was only 35 days and riboflavin was fed at 4, 8 and 12 μ g. per day to groups both with and without atabrine. Similar but less marked differences were obtained (table 1). It appears that the long depletion period with atabrine in the first experiment had limited the ability of the animals to respond.

Protein deficiency

Diets containing 10, 20, and 40% of casein were fed with and without atabrine at a level of 40 mg. %. Over a period of 6 weeks the animals receiving atabrine gained 27, 15, and 19% less than the corresponding control animals. The results appear to be similar to those obtained with animals on riboflavin deficient and complete diets.

Vitamin A deficiency

Thirty-six animals, half of which received atabrine (40 mg. %), were depleted of vitamin A until eye symptoms were noted and the animals began to lose weight (24 days). Groups with and without atabrine were then given daily supplements of 1, 3 and 5 μ g. of carotene

TABLE 2

Average gain in weight on various levels of carotene with and without atabrine in the diet.

MICROGRAMS OF CAROTENE PER DAY	TIME OF EXPERIMENT	NO ATABRINE	PLUS ATABRINE	PLUS ATABRINE AS % OF CONTROLS
	<i>days</i>	<i>grams gain</i>	<i>grams gain</i>	
1	31	15.7	16.1	102
3	39	54.0	58.0	107
5	39	77.4	70.8	91.5

dissolved in corn oil. The gain in weight of the six groups after supplements were started is shown in table 2. There appears to be no growth inhibition due to atabrine except at the highest level of carotene, which is in contrast to the studies on riboflavin and protein.

Reproduction

Three males and three females which had received the basal diet plus 25 mg. % of atabrine from weaning until they were 5 months of age were transferred to a diet of ground commercial dog food^{*} to which was added 25 mg. % of atabrine. They were mated when 6 months

^{*} Purina Dog Chow.

of age. Normal litters of 8, 10, and 6 young have been reared, are now 4 months old and show no abnormalities.

Chick experiments

The chick is much more resistant to atabrine administration than the rat. Five-day-old chicks in groups of six were fed a standard chick mash to which atabrine was added at eight levels from 0 to 240 mg. %. Approximately 160 mg. % of atabrine was required to decrease the rate of gain about 30%. Thus by this criterion the chick can tolerate about four times as much atabrine as the rat. At higher levels of atabrine no effect, other than poor growth and discoloration, were noted. A partially purified adequate diet containing casein at levels of 10, 15, and 20% was fed. As in the rat experiments, the per cent inhibition of atabrine (160 mg. %) was approximately the same at the various levels of protein.

DISCUSSION

It seems clear from these results that atabrine at the levels fed is relatively non-toxic for the rat, and much less so for the chick. In the rat the continuous feeding of a level of 25 mg. per kilogram per day produces no gross symptomatology over a period of 1 year and reproduction is apparently normal. Wright and Lillie ('43) have recently described the pathologic changes produced in rats with high levels of atabrine. The atabrine was administered by stomach tube. At lower levels of atabrine (30 mg. per kilogram, given over a period of seven weeks) little if any effect on growth was noted and only slight pathologic lesions were observed. Care must be used in relating these data to humans, however, since the difference in rats and chicks makes it clear that species vary in ability to handle this drug. Whether the human is more or less tolerant than these species is at present unknown. It probably is significant, however, that we have fed a level of atabrine near that required to inhibit growth for 1 year with no apparent ill effect.

The significance of the effects on deficient diets is not clear. On adequate diets the growth inhibition cannot be overcome by increasing the level of protein or by high levels of vitamins or by yeast. On riboflavin-low or protein-low diets, when the animals are growing slowly, the addition of atabrine decreases the rate of growth still further. Thus they appear to react in the same manner as well nourished animals. In partial vitamin A deficiency, however, the rate of growth

was not decreased by the same level of atabrine used in the previous experiments; it would appear that when vitamin A was the limiting factor atabrine had no effect. On the other hand, the atabrine inhibition in normal animals, or when riboflavin or protein are limiting, may be related to the metabolism of these factors. The metabolism of these is interrelated as shown by Sarett, Klein and Perlzweig ('42). A direct inhibition of riboflavin is improbable since on the riboflavin-low ration and without supplements those animals receiving atabrine did not stop growing or show other signs of deficiency earlier than the controls.

The relatively short depletion period that is required to produce vitamin A deficiency as compared with the riboflavin depletion period may be of importance in the response obtained since less marked effects were observed in the second of two riboflavin experiments when the depletion period was less. However, animals on an adequate diet show a growth inhibition within the time of the depletion period, and a marked growth inhibition over a period as long as the total vitamin A experiment. Preliminary experiments indicate that the maximum level of atabrine in the tissues is reached after a period of from 3 to 4 weeks on a diet containing 40 mg. % atabrine.

CONCLUSIONS

1. Atabrine added to an adequate diet in levels such that the rat receives 25 mg. per kilogram per body weight per day or less is completely non-toxic as judged by growth, general appearance and behavior, gross and micropathology, and reproductive ability.

2. Atabrine levels of 40 to 65 mg. per 100 gm. of ration retarded growth by 20 to 30%; the fur is discolored, and the animals are unkempt. The addition of various vitamins, yeast or protein to an already adequate diet does not prevent these changes.

3. The slow growth obtained on suboptimal levels of riboflavin or protein is further decreased by the addition of 40 mg. % atabrine to the diet. With diets suboptimal in vitamin A addition of atabrine does not cause a further reduction in growth rate.

4. On the basis of rate of growth on diets containing atabrine, the chick is from three to four times as tolerant of atabrine as is the rat.

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THE EFFECT OF ATABRINE ON CHOLINE DEFICIENCY IN THE YOUNG RAT¹

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ONE FIGURE

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Investigations designed to study the effect of various dietary constituents upon the tolerance of rats to the continued administration of the anti-malarial drug atabrine have been reported (Hegsted, McKibbin and Stare, '43). As a part of these studies a low-protein, low-choline diet was fed to weanling rats, with and without atabrine at a level of 40 mg. per 100 gm. of ration. Four of five rats on the control diet died, whereas, to our surprise, none of those receiving atabrine died. This effect of atabrine has been further investigated and is the basis of this paper.

EXPERIMENTAL²

In all experiments weanling male rats purchased from the Rockland Farms have been used. These were placed immediately upon the experimental ration which was fed ad libitum except in one experiment where the food intake of the control group was limited to that consumed by those receiving atabrine. Atabrine was incorporated in the rations by dissolving the dihydrochloride in alcohol and drying on the ration.

Two different diets have been used. The first was low in protein and choline and had the following percentage composition: casein (SMA) 10, lard 20, salt mixture 4 (Hegsted et al., '41), and sucrose 66. Vitamins were added to supply 200 µg. thiamine chloride, 400 µg. riboflavin, 200 µg. pyridoxine, 1.5 mg. calcium pantothenate, 2.5 mg. nicotinic acid per 100 gm. of ration. The fat soluble vitamins were fed by

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

² We are indebted to Merck & Company, Rahway, New Jersey, for furnishing the crystalline B-vitamins and the alpha-tocopherol, and to Abbott Laboratories, North Chicago, Illinois, for furnishing Haliver Oil.

dropper in corn oil to supply 80 I. U. of vitamin A, 1.5 I. U. vitamin D, and 1.0 mg. of alpha-tocopherol per rat per week. The second diet was similar to that used by Engel and Salmon ('41) in studies on choline deficiency. It contained 30% alcohol extracted peanut meal, 6% SMA casein, 6% corn oil, 4% salt mixture (Hegsted et al., '41), and 0.5% K_2HPO_4 . Water soluble and fat soluble vitamins were administered as in the other diet and at the same levels.

RESULTS

In the first experiment using the low protein diet four of the five animals on the control diet died between the tenth and fourteenth day. The animals on the same diet plus 40 mg. of atabrine per 100 gm. of ration showed a slight decrease in weight during this period but then began to gain slowly and gained 32 gm. on the average in the next 2 months. This experiment was repeated using six rats in each group with similar results. Three of the control animals died before the fourteenth day at which time all of the animals were killed and their kidneys weighed and examined for gross lesions. Only slight damage was apparent in the atabrine group and the average weight of the individual kidneys was 260 mg. compared to 353 mg. for the controls without atabrine. Practically no growth was obtained during the first 2 weeks of the experiment. The average weight of both groups at the beginning was 30.6 gm.; those in the atabrine groups weighed 32.8 gm. at the time they were killed compared to 30.7 for the controls remaining on the fourteenth day.

Practically the same results were obtained using the peanut meal ration which is adequate except for choline. Four of five animals died on this ration of choline deficiency within 10 days while no deaths occurred when atabrine was incorporated in the ration at a level of 40 mg. %. The detailed results of the second experiment with this diet are included in table 1. All of the animals were killed on the seventh day when the first animal in the control group became moribund. The kidneys were weighed and examined for gross lesions, and the liver fat determined in the dried livers by chlorform extraction. At this early date some of the animals had not yet developed gross symptoms and the differences are not as great as might be expected had the animals been continued for a few days longer. Only slight protection was noted in the group receiving atabrine at the 40 mg. % level. It seems probable, however, from the previous experiment that these animals would have survived the critical period of choline

TABLE 1

Data obtained on rats fed a low choline ration with various supplements.

SUPPLEMENT	TIME ON EXPERIMENT	AVERAGE WEIGHT OF ANIMALS	AVERAGE WEIGHT OF KIDNEYS	KIDNEY WT. AS % OF BODY WEIGHT	ANIMALS WITH HEMORRHAGIC KIDNEYS	LIVER FAT
	days	grams	mg.		%	% dry wt.
None	7	47.5	343	1.44	60	37.4
40 mg. % ¹ atabrine	7	44.2	359	1.63	40	37.2
65 mg. % ¹ atabrine	7	43.9	284	1.28	0 (?)	43.7
200 mg. % ¹ choline	7	59.0	356	1.21	0	13.0
None ²	12	54.0	616	2.21	83	33.4
65 mg. % ¹ atabrine	12	57.3	414	1.53	33	44.5

¹ Mg. per 100 gm. of ration.

² Food intake limited to the amount consumed by the animals receiving 65 mg. % atabrine in the diet.

deficiency (Griffith, '41). At the higher level of atabrine the protection was practically complete although a few showed slight signs of kidney damage. Atabrine appeared to have no lipotropic action.

Since Griffith ('41) has shown that limiting the food intake will decrease the severity of kidney damage, this experiment was repeated and the food intake of the atabrine group was measured and that of the control group limited to this amount. As the control animals died their kidneys were examined and weighed, and the livers dried for fat analysis. All of the remaining animals were killed on the twelfth day. The growth curves for these groups are shown in figure 1 and the details of our analyses in table 1. Contrary to our experience with adequate diets (Hegsted, McKibbin and Stare, '43), the efficiency of

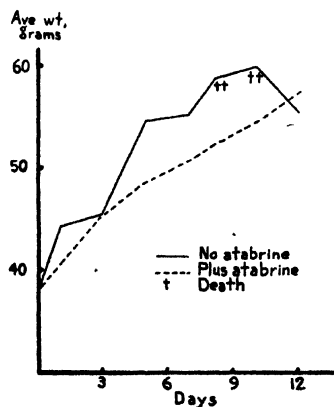


Fig. 1 Growth curves for animals on choline-low diet with and without atabrine at 65 mg. per 100 gm. of ration.

food utilization was somewhat impaired by atabrine administration. Four of the five animals on the control diet showed severe kidney damage and the kidney weights of these four were well above 2% of the body weight. Only two of the animals receiving atabrine had gross kidney lesions and in only one of these was the kidney weight in excess of 2% of the body weight.

DISCUSSION

Moyer and du Vigneaud ('42) have summarized the data upon those compounds which have a choline-like action in the chick and rat. The action of all such compounds reported to date can be explained upon the basis of transferable methyl groups or by the replacement of choline directly (arsenocholine, triethylcholine). The action of dimethylaminoazobenzene as a methyl donor has been reported by Jacobi and Baumann ('42) and indicates that the list of compounds with available methyl groups may be extensive indeed. The availability of methoxyl groups, the only source of methyl groups in atabrine, has not yet been reported and must await further investigation. Two N-ethyl groups are present in atabrine. Since triethylcholine prevents hemorrhagic kidneys, one might speculate on the possibility of these groups acting to replace methyl groups. However, there appears to be no evidence that transeethylations occur in the body and it appears more likely that triethylcholine acts directly by replacing choline.

In their review on tissue changes in vitamin deficiencies Wolbach and Bessey ('42) mention, "Although the mechanism underlying the hemorrhages is not clear, the function of acetylcholine as a neuromuscular mediator is suggestive of a neurovascular cause." This suggests that those drugs affecting the choline esterase system might play a role in modifying the kidney lesions. This phase of the problem is being investigated.

Finally the part played by food intake and growth must be considered. By the paired feeding method our results were as clear cut as with ad libitum feeding. However, the control animals gained somewhat more on the limited food intake than the animals receiving atabrine on the peanut meal ration. On the low-protein ration the gains for the animals with and without atabrine were practically the same although the food intake was not measured. If these rather slight differences are the determining factor, a great deal of care must be exercised in studies on compounds being tested for choline-like action.

The average food intake of our rats on the peanut meal ration plus 65 mg. % atabrine was from 5 to 5.7 gm. per day over the 12 days

on experiment. This represents an atabrine intake of 3.2 to 3.7 mg. per day which is more than required to prevent death on this choline-low ration. Engel states that approximately 5 mg. of choline is required on a similar ration to allow complete survival. This indicates a high degree of activity in view of the fact that atabrine has a molecular weight approximately three times that of choline, although no direct comparison has been made on our ration.

CONCLUSIONS

The inclusion of atabrine in low choline diets at a level of 65 mg. per 100 gm. of ration almost completely prevents hemorrhagic kidneys in the rat. A level of 40 mg. per 100 gm. prevents death but allows some kidney damage to develop. No lipotropic action was observed at the levels fed.

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VITAMIN A STORAGE AND FACTORS THAT AFFECT THE LIVER¹

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ONE FIGURE

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While the storage of vitamin A in the liver depends primarily upon the amount of vitamin absorbed from the digestive tract, this storage can also be modified by certain agents that affect the liver itself. Ethyl alcohol (Ikegaki, '38), thorotrast (Lasch and Roller, '36) bismuth (Wendt and Koenig, '37), and carcinogens such as 1, 2, 5, 6-dibenzanthracene (Goerner and Goerner, '39) are all reported to hasten the depletion of hepatic stores of the vitamin, and it has been suggested (Baumann, Foster and Moore, '42) that the hydrocarbon acts by interfering with a combination of vitamin A and protein or some other "anchor" in the liver. However, not all metabolic changes in the liver are accompanied by changes in hepatic vitamin A. In starvation the glycogen content of the liver diminishes without any parallel loss of the vitamin (Dann and Moore, '31). Phosphorus (Lasch, '35) is reported to produce degeneration of the liver without effect on vitamin A storage. Abels et al., ('42) concluded that the storage of vitamin A is independent of general hepatic dysfunction. The present experiments deal with the hepatic storage of vitamin A in rats or mice fed substances that in one way or another modify biochemical reactions taking place in the liver.

METHODS

All experiments were performed in series involving groups of animals comparable in weight, sex and previous vitamin A intake. Initial stores of the vitamin were established by feeding halibut liver oil. The various groups within the series were then fed diets free from vitamin A but involving other modifications. After a suitable period of depletion, usually 6 or 8 weeks, the animals were killed for analysis and the vitamin A content of the liver determined colorimetrically (Davies, '33;

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Baumann, Riising and Steenbock, '34). The basal diet deficient in vitamin A (diet I) consisted of heated casein 18%, salts 4%, yeast 7.9%, irradiated yeast 0.1%, agar 2%, cottonseed oil 5% and dextrinized starch 63%.

EXPERIMENTAL

p-Dimethylaminoazobenzene and hepatic storage of vitamin A

In a previous experiment colloidal suspensions of dimethylaminoazobenzene were injected intraperitoneally for 8 weeks into rats fed diet I (Baumann, Foster and Lavik, '41) with practically no effect of the dye on the vitamin although the carcinogenic hydrocarbons markedly reduced the storage of vitamin A under these conditions. Diet I, however, also tends to minimize the production of liver tumors due to *p*-dimethylaminoazobene (Miller et al., '41).

In the present experiments the rate of depletion of vitamin A was measured under more optimal conditions for the production of liver tumors with the azo dye (Miller et al., '41). The basal diet deficient in vitamin A was also low in riboflavin and it was not particularly high in protein (diet II). It consisted of 12% of heated casein, 2% of rice bran concentrate, 4% of salts, 5% of cottonseed oil and 77% of dextrin. Dimethylaminoazobenzene was added to the cottonseed oil as 0.06% of the diet.

Twenty rats, averaging 93 gm. in weight, were each fed 2 drops of halibut liver oil to establish moderately high stores of vitamin A. Two rats were killed 3 days later and the vitamin content of the livers found to average 502 μ g. of vitamin A per rat. The remaining rats were then divided and the first group fed diet II ad libitum. A second group was fed the same diet ad libitum, but minus the azo dye (diet III), while a third group was fed diet III in the amounts consumed by the group getting the carcinogen. The effect of the dye was also determined on two other groups fed diet I with and without the carcinogen. Six weeks later all rats were killed for analysis.

No pronounced variation in vitamin A storage resulted from the feeding of the azo dye. After 6 weeks of depletion the hepatic stores averaged 374 μ g. of vitamin A per liver on the carcinogenic low A diet, (diet III) as compared to 355 μ g. in the presence of the azo dye (diet II). On diet I, which tends to minimize tumor formation, the figures were 386 μ g. in the absence of the dye as compared to 353 μ g. in its presence. These differences in averages were not considered significant. The slight lowering of hepatic vitamin A in the groups fed the dye was probably caused by a reduced consumption of food rather than by the dye itself,

since the group on the restricted food intake contained the same amounts of hepatic vitamin A as the group fed the dye. Certainly the effect of the azo dye was very much less than the effects previously observed with alcohol or with the carcinogenic hydrocarbons (Baumann et al., '41, '42). Extracts of the combined organs (lungs, kidneys, adrenals, spleen and blood) from animals receiving the dye yielded a red color with SbCl_3 .

TABLE 1

The effect of p-Dimethylaminoazobenzene on hepatic vitamin A.

DIET	AZO DYE	WEEKS ON EXPT.	AVERAGE WEIGHT OF RATS	VITAMIN A PER LIVER	NO. OF RATS
	%		gm.	$\mu\text{g.}$	
I — ad lib.	0	0	106	502 (436-604)	2
I — ad lib.	0	6	179	386 (359-408)	3
I — ad lib.	.06	6	133	353 (346-361)	2
II — ad lib.	.06	6	94	355 (343-380)	5
III — ad lib.	0	6	129	374 (365-392)	3
III — restricted	0	6	102	354 (330-395)	3

Diet I contained 18% of casein and 8% of yeast. Liver tumors do not develop readily on this diet.

Diet II contained 12% of casein and 2% of a rice bran concentrate. On this diet the incidence of liver tumors frequently reaches 100% in 4 to 6 months. Diet III is the same as Diet II except that the dye is omitted.

3, 3'-methylenebis (4-hydroxycoumarin) and hepatic vitamin A

It is generally believed that prothrombin is synthesized in the liver (Smith, Warner and Brinkhous, '37). The anticoagulant 3, 3'-methylenebis (4-hydroxycoumarin)² causes a hypoprothrombinemia when fed to animals, although it does not affect the clotting of blood in vitro (Campbell et al., '41). Vitamin K on the contrary tends to increase prothrombin activity, and in the rat, at least, can be used to overcome the effects of the anticoagulant (Overman et al., '42). Carvone, which stimulates the synthesis of vitamin C in the rat, also counteracts the effect of the anticoagulant (Baumann, Field, Overman and Link, '42). In the present experiments the anticoagulant, vitamin K, carvone and combinations of these compounds were added to the basal diet I low in vitamin A and their effect on hepatic vitamin A determined.

Twenty-six rats, 44 gm. in weight, were given 3 drops of halibut liver oil at the beginning of the experiment and the various low vitamin A diets (table 2) fed for 8 weeks. The anticoagulant was fed as a mixture

² We are indebted to Prof. K. P. Link for this preparation.

of 1 mg. incorporated into 1 gm. of diet I. This amount fed three times a week to the young rats resulted in pronounced hemorrhages in the subcutaneous areas of the face or from the nose, and four of nine animals died. Accordingly the dosage of anticoagulant was reduced to 1 mg. a week and gradually increased to the desired level as the surviving rats increased in weight.

As a source of vitamin K 50 mg. of 2 methyl 1, 4 naphthoquinone were added per kilogram of diet I. The carvone was mixed with the low vitamin A ration at the expense of the dextrin in the amount of 2.5 gm. of carvone per kilogram of ration. No gross abnormalities were evident in any of the animals on the latter diets.

TABLE 2

The depletion of hepatic vitamin A in rats fed substances that affect prothrombin activity.

SUPPLEMENT TO A-LOW DIET I	NUMBER OF RATS	WEEKS ON EXPT.	WEIGHT OF RATS	DICUMAROL ¹ PER RAT	VITAMIN A PER LIVER
				mg.	μg.
None	2	0	48	728 (658-798)
None	4	8	207	307 (270-364)
Dicumarol ¹	6	8	187	14-15	347 (264-484)
Vitamin K	2	8	163	350 (340-360)
Dicumarol ¹ and Vitamin K	2	8	164	9-10	347 (336-357)
Carvone	3	8	174	313 (244-380)
Carvone and Dicumarol ¹	3	8	169	14	255 (243-267)

¹ 3, 3'-methylenebis (4-hydroxycoumarin).

Immediately after the ingestion of halibut liver oil, the animals averaged 728 μg. of vitamin A per liver. Control animals kept for 8 weeks on diet I, with no variations, retained 307 μg. of the vitamin in their livers at the end of this period. Animals receiving the 3, 3'-methylenebis (4-hydroxycoumarin) or vitamin K or a combination of these compounds retained 350 μg. of vitamin A per liver (table 2). Thus neither the anticoagulant nor vitamin K hastened the depletion of the hepatic vitamin A; in fact if anything, they appeared to retard it somewhat. Since both the anticoagulant and vitamin K affected vitamin A storage in the same way, it was evident that there was no parallelism between vitamin A storage and prothrombin activity. Carvone also appeared to be without effect upon vitamin A storage (table 2).

Effect of fat and choline

In the first series eighteen female rats 145 gm. in weight (± 20) were placed on three different rations low in vitamin A. Twelve of the

rats were fed a high fat diet IV consisting of extracted casein 5%, cystine 0.2%, yeast 5%, salts 4%, cottonseed oil 2%, hydrogenated cottonseed oil 30%, and dextrin 53.8%. Three other rats received the above ration supplemented with 0.3% choline. The remaining three rats were fed diet V which was low in fat as follows: casein 3.5%, cystine 0.15%, yeast 3.5%, salts 2.9%, cottonseed oil 1.5% and dextrin 88.5%. After 1 week each rat was given 2 drops of halibut liver oil, and 2 weeks later two of the animals on diet V were killed for analysis. Five of the other rats were continued on diet IV while the remaining five were fed the low-fat diet supplemented with 0.3% choline (diet VI). At the end of the fourth week all animals in the series were killed for analysis. For the quantitative determination of vitamin A the livers were allowed to stand overnight in alcoholic KOH at room temperature

TABLE 3

The effect of fat and of choline on the hepatic storage of vitamin A.

DIET	NUMBER OF RATS	WEEKS ON EXPERIMENT	WEIGHT BEGIN.—END	PER CENT LIVER FAT	VITAMIN A PER LIVER
			<i>gm.</i>	<i>fresh wt.</i>	<i>μg.</i>
High fat diet IV	2	3	147-164	31	337 (312-362)
High fat diet IV	5	4	150-156	40	381 (289-478)
High fat IV + 0.3% choline	3	4	148-159	3.4	395 (278-478)
Low fat diet V	3	4	149-157	24	311 (291-342)
High fat IV, 3 weeks, low fat + choline, diet VI 4th week	5	4	147-163	27	370 (286-518)

followed by 30 minutes on the steam bath. This procedure resulted in a more thorough saponification of the fatty livers in the series. Previous analyses had indicated that the values for vitamin A in very fatty livers are increased by 15% when a hot alcoholic saponification is employed in place of the usual cold aqueous solution of the tissue (Clayton, '42).

Except in the group fed choline throughout the experiment, the animals had typically enlarged, pale, and friable fatty livers. Rats fed the high fat diet IV for 4 weeks had a liver fat content of 40% based on the fresh weight, in contrast to a percentage of only 3.4% in the group fed an additional 0.3% of choline. Nevertheless both groups contained essentially the same amount of hepatic vitamin A (table 3) and in the entire series the variations in hepatic vitamin A within the various groups were usually greater than between groups.

The relationship between liver fat and hepatic vitamin A was also studied in ninety adult mice that were fed either a diet comparatively low in fat (diet I) throughout the experiment or a pair of diets alternating at weekly intervals. One of the latter diets was designed to effect a rapid accumulation of fat in the liver, while the other caused a rapid rate of depletion of the surplus liver fat. Uniform storage of hepatic vitamin A was assured by feeding 1 drop of halibut liver oil to each mouse at the beginning of the experiment. Thereafter all rations fed were free from vitamin A. Diet VII was high in fat and low in lipotropic factors, yet contained as much total protein as the

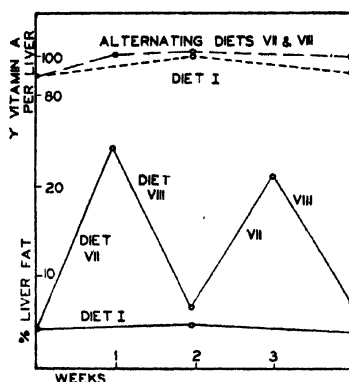


Fig. 1. Constancy of vitamin A in mouse livers of varying fat content.

The solid lines represent the percentages of liver fat; the broken lines, the micrograms of vitamin A per liver. Each circle represents the averages of at least five determinations made separately.

Diet VII was high in fat and low in lipotropic factors, viz. casein 5%, arachin 13%, cystine 0.2%, fat 32%, etc. Diet VIII was low in fat and high in lipotropic substances: casein 18%, choline 1%, etc. Diet I contained 18% of casein and 5% of fat.

other low A diets fed. It consisted of casein 5%, arachin 13%, cystine 0.2%, salts 4%, yeast 5%, hydrogenated vegetable oil 30%, cottonseed oil 2%, and dextrin 40.8%. Diet VIII, fed for the rapid removal of fat from the liver, consisted of diet I plus 1% of choline, the lipotropic effect of which was enhanced by the 18% of casein also present in the diet. Diets VII and VIII were fed alternately at intervals of 1 week.

The results are presented in figure 1. On the low fat diet I, the liver fat remained at approximately 4% of the fresh weight during the 4 weeks of the experiment. In contrast, the group fed diet VII (arachin) developed very fatty livers within 1 week (liver fat = 24%), and when diet VIII was fed to these animals most of the extra fat had disap-

peared from the liver 1 week later. Subsequent feeding of the arachin diet raised the percentage of liver fat to 21% and this extra fat was again removed from the livers after feeding diet VIII for 1 week longer. In spite of these very drastic changes in the percentage of liver fat, the vitamin A content of the liver remained essentially unchanged during the 4-week experiment. Furthermore, the amount of vitamin A in the livers of mice fed the alternating diets was essentially the same as in those fed diet I throughout the experimental period.

In agreement with Lease and Steenbock ('39) and contrary to the results of Thorbjarnson and Drummond ('38) our results all appeared to indicate that the retention of vitamin A in the liver did not depend upon the retention of fat by this organ. Since choline is very active in removing fat from the liver, it might be inferred that choline likewise has no effect on hepatic vitamin A. However, the experiments both with rats and mice were performed with adult animals, which are known to be relatively resistant to the effects of choline deficiency *per se* (Griffith, '40). Accordingly, the distribution of vitamin A was determined in young rats exhibiting very severe symptoms of choline deficiency.

Eighteen rats, 31–39 gm. in weight, were each fed 2 drops of halibut liver oil. They were then placed on a synthetic diet free from choline (Jacobi and Baumann, '42) and six of the animals received an additional 8.5 mg. of choline per day. On the second day three of the animals were killed for analysis of the vitamin A content of the liver, kidney, and other organs. After 5 days the animals on the low choline diet began to lose weight, and four of nine developed severe intraocular hemorrhages. On autopsy all of the deficient animals had enlarged kidneys, frequently bloody or mottled. In contrast the animals receiving the choline supplement remained in good physical condition, and no abnormalities of the kidneys were evident. In spite of these differences between groups the vitamin A contents of both livers and kidneys were essentially the same in the presence or absence of dietary choline (table 4). Contrary to the report of Popper and Chinn ('42) there was no evidence that the choline deficiency had altered the distribution between the liver and kidney of preformed stores of vitamin A. Evidence of a shift of vitamin A from the liver to the kidney may however have been suggested in another group of eight rats, 50 gm. in weight, fed the low choline diet (\pm choline) for 10 days plus 50 μ g. of carotene per rat per day fed by dropper in cottonseed oil. The animals in both groups averaged 77 gm. in weight after 10 days without any visible symptoms of choline deficiency. Those on the deficient diet contained

only occasional traces of the vitamin per liver while 3.5 μ g. of the vitamin were present per pair of kidneys. In contrast, the animals receiving choline averaged 3.9 μ g. of vitamin A per liver and only 1.6 μ g. per pair of kidneys. These amounts of vitamin A, however, were not much greater than the limit of error of the analytical method employed, and furthermore, the translocation of the vitamin was considerably less than that previously observed in the presence of dibenzanthracene (Baumann, Foster and Moore, '42).

TABLE 4

The distribution of vitamin A in the tissues of choline-deficient rats.

DIET	DAY	MICROGRAMS VITAMIN A			NUMBER OF RATS
		Liver	Kidneys	Organs ¹	
	2	260	3.5	2.7	3
Low choline	10	278	9.1	4.2	6
High choline	10	234	8.0	4.0	3
Low choline	20	234	5.3	1.8	3
High choline	20	233	6.0	2.3	3

¹ The organs included the lungs, spleen, adrenals and approximately 2 ml. of blood from each rat.

DISCUSSION

The hepatic storage of vitamin A was found to be unaltered by the most potent carcinogenic agent known for the liver of the rat; it was unaffected by factors that either depress or enhance the formation of prothrombin; and it remained essentially the same whether the diets fed were high or low in fat. Large amounts of fat could be removed from the liver without any accompanying loss of vitamin A from this organ. Nor did the retention of vitamin A by the liver appear to depend upon the presence of choline in the diet. These results all suggest that vitamin A is stored in the liver and released therefrom by a mechanism that is comparatively independent of other processes taking place in this organ. A similar conclusion is implicit in the results of Abels et al. ('42), who observed that dibenzanthracene, which seriously interferes with the retention of vitamin A by the liver, failed to affect the esterification of cholesterol, the formation or the conjugation of glucuronic acid, the formation of prothrombin or of other proteins of blood plasma, or the storage capacity of the liver for total fat, phospholipid or riboflavin. It would thus appear that many chemical reactions proceed in the liver independently of one another, and that a mild derangement of one does not necessarily interfere with the others.

In severe hepatic failure, on the contrary, it would obviously be possible for more than one process to be affected.

SUMMARY

The hepatic storage of vitamin A appeared to be relatively independent of other biochemical processes taking place in the liver. *p*-Dimethylaminoazobenzene, a potent agent for the production of liver tumors in the rat, failed to alter the rate of depletion of preformed stores of the vitamin, even on diets optimal for the carcinogenic action of the dye.

3, 3-Methylenebis (4-hydroxycoumarin), which depresses prothrombin synthesis, and vitamin K, which promotes it, were equally without effect on the rate of depletion of hepatic vitamin A. Carvone, likewise, was without effect.

The retention of vitamin A by the liver of the rat was essentially the same on diets either very high or low in fat; the rate of depletion of the vitamin from very fatty livers was the same as that from normal livers. In the mouse very high percentages of liver fat were repeatedly accumulated and "flushed out" without effect on the retention of hepatic vitamin A. The normal distribution of vitamin A was observed in tissues of young rats with severe symptoms of choline deficiency.

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PHYSIOLOGICAL AND BIOCHEMICAL FUNCTIONS IN NORMAL YOUNG MEN ON A DIET RESTRICTED IN RIBOFLAVIN ¹

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In this laboratory we have been concerned with the possible limitation of human work performance by restriction in dietary elements such as may be encountered in war time, particularly in military operations. The vitamins of the B complex have been prominently mentioned in this connection in certain circles. Of these vitamins thiamine, riboflavin and niacin occupy first place by nature of their undoubted importance in fundamental enzyme reactions involved in carbohydrate and presumably muscle metabolism.

We have previously reported our inability to detect any advantage for work performance of "supercharging" with these vitamins over periods of 6 to 10 weeks (Keys and Henschel, '42). Studies on young men maintained for 10 to 12 weeks on considerably less than half the National Research Council recommended allowance for thiamine failed to disclose any limitation on work performance and related metabolic functions (Keys, et al., '43). The present article is a report on similar studies with an intake of riboflavin restricted to about one-third the N.R.C. recommendation for a period of 22 weeks with additional control periods totaling 15 weeks.

SUBJECTS AND REGIME

The subjects were six men students free from signs and histories of metabolic, nutritional and digestive peculiarities. They were especially selected for reliability and cooperation. During the entire experimental

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period with the exception of a few pre-arranged days and a part of one control period the subjects received all meals in the laboratory and resided in rooms nearby. They were not under immediate supervision except at meal times and 1 or 2 days each week but they were thoroughly aware of the importance of conforming to the limitations of the program and were undoubtedly anxious to fulfill their roles as cooperators. Their adherence to the conditions was demonstrated by the results of urine analyses which were made weekly and by the reports of their classmates.

Once each week on the average the 24-hour urine was collected and from time to time this was succeeded by a 24-hour "saturation test" collection. Once each week the subjects carried out a series of work tests which involved, successively, 30 minutes' bed rest, 60 minutes' walk on the treadmill at 3.85 miles per hour and 10% grade, 2 minutes' standing rest, 10 minutes' bed rest, 60 seconds' run on the treadmill at 9.5 miles per hour and 10% grade, bed rest for 25 minutes. Every other week venous blood samples were taken at the end of the first bed rest and at 8, 12 and 25 minutes after the end of the work in the final bed rest. Pulse rates were taken during the first bed rest, during the final 10 minutes of the walk, for the first 2 minutes (standing) after the walk, and at 1 and 2 minutes after the end of the run. On alternate weeks the battery of psychomotor tests was run through once during the first 10 minutes of walk on the treadmill and again in the last 10 minutes of the walk. All test procedures were made in a controlled environment suite, temperature 78°F., humidity 50% relative saturation. Standard clothing in all tests consisted of cotton gymnasium shirts and shorts, socks and shoes.

Clinical examinations were made each month; these included slit lamp observations of the corneas and very careful examination of the skin, lips, mouth and tongue. Electrocardiograms and measurements of basal metabolism were made before and after as well as at intervals during the restricted regime.

All subjects were trained and standardized with the several tests and procedures for 3 weeks preceding the restricted diet period. During this time the men subsisted on the ordinary diet furnished by their boarding houses. Three of the men (group A) were maintained on a restricted diet for 84 days, and the other three men (group B) were continued on for a total of 152 days on the low B₂ intake followed successively by 26 days on a high (11 mg.) intake, 6 days on the low regime, 36 days on a relatively normal B₂ intake and finally 8 days on the low intake.

DIET

All the meals were prepared in the laboratory diet kitchen by a dietitian and assistants. Strict menus were followed and all portions were weighed. An extra identical meal was prepared and used for analysis. These were rapidly and carefully ground and stored at -25°C . for daily analysis.

The basic composition of the diet provided a "normal" balance — 55 to 75 gm. (av. 60 gm.) of protein and 37% of the calories as fat. Milk and eggs were not used and only very small amounts of meats and green vegetables were allowed. Unfortified flour was used for all breads and pastries. Daily supplements, in capsules, provided 1.0 mg. thiamine, 10 mg. niacinamide, 50 mg. ascorbic acid. The total daily caloric intake was first set at 3000 Cal. This proved slightly less than required to maintain body weight so the amount was increased, by additional breads, carbohydrates and a small amount of fat, to an average of 3160 Cal.; these additions sufficed to maintain body weights in all but one subject, C. M., who lost 12 pounds (177 to 165) in the 5-month period on the low B_2 regime. The final average caloric intake then was about 3150 ± 100 Cal.

The riboflavin content of the diet was planned to be about 1.0 mg. per day or slightly less. Actually, the analyses for the first 3 weeks indicated a riboflavin content about 20% greater than calculated from the available "book" values. Accordingly the diet was then more severely restricted so that the average daily intake for the entire 152 days was 0.99 mg. or a grand average of 0.31 mg. of B_2 per 1000 Cal. The riboflavin intakes for different periods are indicated in table 1. Foods for representative days are given in table 6.

METHODS

Great pains were taken to ensure the validity of all riboflavin analyses. Riboflavin in the urine was estimated both by the microbiological method of Snell and Strong ('39) and by a modification of the fluorometric method of Connor and Straub ('41). The latter procedure proved to be considerably more satisfactory and was relied upon exclusively for the last 3 months. For the foods the microbiological method of Snell and Strong was modified according to the nature of the material, the criteria being the production of a crystal clear filtrate, the recovery of known additions, and good agreement between duplicates (Andrews, et al., '42). Ether extraction, diastatic treatment and filtration at pH 4.5 were variously applied to achieve these ends.

The 24-hour urine was collected in amber glass bottles containing 5 ml. of glacial acetic acid and 3 ml. of toluol. The samples were stored at 3°C. while awaiting analysis.

Venous blood, drawn from a vein in the antecubital fossa with a minimum of stasis, was analyzed for lactate by the method of Friedemann, Cotonio and Shaffer as modified by Edwards ('38). Pyruvate was estimated by the method of Lu ('39) as modified by Friedemann and Haugen ('43). Blood sugar was measured by the method of Folin and Wu ('20) using cadmium hydroxide as the precipitating agent. Hemoglobin was estimated as oxyhemoglobin with a photoelectric colorimeter.

Measurements of flicker fusion frequency and of grip and back strength were made before and after the 60-minute walk periods. Manual speed was tested with Brozek's ('43) ball and pipe method, motor control by tracing a stylus through a "maze," and speed of small movements by the tapping test as used previously (Keys, et al., '43). These last three tests were carried out during actual work on the treadmill.

RESULTS

The data are too extensive for detailed presentation here. Fortunately there was relatively little individual variation between the several subjects so treatment by averages is justifiable as well as expedient. The two groups were closely similar in all respects up to the time of discontinuance of group A so only the measurements with group B will be given in some of the tables. It must be emphasized, however, that the data cited on group A could apply almost identically to group B up to the end of their participation (84 days on the restricted diet).

Riboflavin excretion

Riboflavin excretion in the 24-hour urine for the restricted period is summarized in table 1.² During the first few weeks on the restricted regime the riboflavin excretion decreased progressively but after about a month and a half the excretion appeared to be stabilized at from 10 to 14% of the intake. When extra test doses were introduced their per cent of recovery was very similar. The indication of slightly smaller recovery of test doses in the table cannot be given any weight because of the technical difficulty of the procedure.

² Results from one subject (E.L.F.) are omitted because we were consistently baffled in the attempt to get reliable results on his urine; in particular the microbiological and chemical methods were frequently in disagreement on his urine.

When, after 5 months on the restricted regime, the daily intake of riboflavin was raised to 11.2 mg., the urinary excretion increased abruptly so that after 1 week an average of 54% (45, 58 and 62% in the three men tested) of the intake appeared in the urine. Return to the low riboflavin intake after 26 days of this supercharging brought the excretion down to an average of 38% of the intake on the fifth day. A saturation test on the sixth day showed 19.7% recovery. Finally, return to fraternity house diet for 5 weeks resulted in excretions of 30 to 100 micrograms of riboflavin per day after an additional week on a measured daily intake of 0.95 mg.

TABLE 1

Average riboflavin intake and 24-hour excretion. Intake values are averages, in milligrams per day, for the days indicated. Excretion values, in micrograms in 24 hours, are the averages of two to four measurements for each man in the indicated periods. "Test recov." shows the elevation of the 24-hour excretion resulting from administration of 1.0 mg. of crystalline riboflavin.

Days	4-8	15-22	28-38	48-55	63-72	78-85	97-104	110-119	126-134	142-149
Intake	1.32	0.97	1.04	0.98	1.02	0.90	0.87	0.89	0.82	0.90
Excretion:										
Subj. C M	...	155	155	147	120	139	162	87	98	108
Subj. L E	422	263	251	189	145	182	119	108	112	123
Subj. C T	359	175	149	101	81	96	77	62	98	89
Subj. F T	230	294	179	188	159	153
Subj. G W	213	186	135	81	83	82
Average	308	215	174	141	118	130	119	89	103	107
As % intake	23%	22%	17%	14%	12%	14%	14%	10%	12%	12%
Av. test recov.	9.1%	13.4%	8.0%

The data clearly show that the body can accumulate a reserve of riboflavin but that this reserve was not great and was lost in a few weeks. It is important that, in the present series, an equilibrium between intake and output was established on the low regime and there were no signs of progressive alteration in this equilibrium after the first 2 or 3 months.

Eye examinations

Five out of the six subjects initially showed minor peculiarities in the slit lamp examinations. These included whitish, granular, sub-epithelial deposits at the limbus and the center of the cornea and slight peripheral vascularization at the limbus (4 to 5 arcades, 3 to 8 mm.). There was no change in any of the eyes at any time during the period of the restricted riboflavin intake nor afterwards in the restoration control periods. In all other respects also the eyes were apparently unaffected by the diets.

Other clinical observations

The skin, mouth and tongue of all subjects remained apparently entirely normal at all times. No signs of cheilosis, stomatitis or scaling at the nasolabial folds appeared. All other observations were constantly negative. At no time was there any evidence of alteration in gastro-intestinal, cardiovascular or neuromuscular functions. The electro-cardiograms were unchanged in all men in all leads throughout the entire period of observation. The direction, potential and duration of the several components of the E.C.G. were examined.

Work performance

In any work performance measurements, it is essential that the work tasks and conditions be rigidly standardized and the training factor must be held constant. In the present study it was not possible to maintain absolute constancy of general muscular training and rest. These studies were started at the end of the summer when these subjects, like college students in general, were in a better state of physical training than at other times of the year. About a month after the start of the low B_2 regime the subjects began to "cram" for their comprehensive examinations with the result that recreational muscular exercise and hours of sleep were cut to a minimum for about a month. The effects of these conditions were reflected in some of the measurements in the performance studies.

The heart rate in standard rest was remarkably constant throughout. The heart rate in work, because of its central position in the homoeostatic-mechanisms of the body, is a sensitive and valuable criterion of work capacity. In the 60-minute walk difficulties with the cardio-tachometer spoiled the pulse rate records in work for the initial control period and the first few weeks on the low B_2 intake. Thereafter, however, the rates were substantially constant for about 2 months with a grand average of 141 and weekly maximum and minimum of 144 and 137 beats per minute. Several weeks after the end of the comprehensive college examinations there was a slight improvement in all men and this new level of work pulse rate (average 131) was maintained with substantially no change for the balance of all the studies, including those in the "restoration control" period.

The average heart rates for group B in recovery following anaerobic work are given in table 2. It is clear that after about a month on the low B_2 regime the men exhibited a moderate decline in capacity for this type of work which persisted until the end of the college examination period

and subsequently there was small improvement in the 1-minute pulse and a more marked return toward the original values in the 2-minute pulse. There were no changes in the "restoration control" period.

Average values for blood glucose, lactate and pyruvate in rest and after work in group B are given in table 3. The glucose and pyruvate values were remarkably constant in all periods but there are some

TABLE 2

Average pulse rates, group B, after 30 minutes' bed rest and at 1 and 2 minutes after standard anaerobic work. "Before" represents the control period before B₂ restriction and after" is the restoration control after 17 days on a daily intake of 11 mg. of B₂. Other column headings refer to the number of days on the restricted regime.

CONDITION	BEFORE	22	36	50	64	78	105	119	134	148	AFTER
Rest	66	67	68	65	69	68	69	71	69	66	72
1 Min.	142	145	159	159	166	153	153	155	151	152	160
2 Min.	132	133	148	152	145	135	131	137	133	136	136

TABLE 3

Average values, group B, for blood analyses in rest and at 12 and at 25 minutes after standard anaerobic work. All values in ml. per 100 ml. of whole blood. "Before" represents the control period before B₂ restriction. "After" represents the "restoration" control after 17 days on a daily intake of 11 mg. riboflavin. The days noted refer to number of days on the restricted regime.

PERIOD	REST			12 MIN. RECOVERY			25 MIN. RECOVERY		
	Glucose	Lactate	Pyruvate	Glucose	Lactate	Pyruvate	Glucose	Lactate	Pyruvate
Before	79	9	0.9	74	47	3.5	75	43	2.5
22 days	81	8	0.8	75	42	2.5	72	31	2.3
36 days	73	14	0.7	78	48	2.5	76	33	1.9
50 days	76	14	0.7	74	55	2.7	68	41	1.9
64 days	82	13	1.0	76	55	2.7	72	44	1.9
78 days	79	12	0.9	80	62	3.2	81	39	2.4
105 days	79	10	0.8	76	64	3.0	73	35	2.4
119 days	76	7	0.8	75	57	3.1	71	34	2.2
134 days	78	7	0.7	78	61	3.4	75	38	2.5
148 days	79	7	0.9	78	56	3.6	71	38	2.5
After	79	7	1.0	81	55	3.5	73	28	2.6

points to be noted in the lactate concentrations. There was a slight elevation in the resting lactate from the thirty-sixth to the seventy-eighth day; this period covers the "cramming" and examination period and several weeks afterwards. We have noted the temporary disturbance in the pulse rates in this period. A slightly greater lactate accumulation at 12 minutes after work appeared after about 6 weeks on the

restricted regime. This change was small and not progressive. Its explanation is questionable but presumably it reflects a loss of the benefits of the summer physical activity.

The results of the simple muscle strength tests are notable for their really surprising constancy. For example, the grip strength of one subject (C. T.) did not vary from week to week beyond the range of 41 to 44 pounds and that of another subject (C. M.) beyond the range of 66 to 72 pounds. The weekly averages of grip strength for group B varied from 51 to 54 pounds in four preliminary control periods, from 52 to 56 pounds in the entire restricted diet period and from 53 to 54 in the "restoration control" period. This is interesting in view of the fact that there is evidence that grip strength responds to slight changes in condition, incipient colds etc., (Metheny, '40). The back dynamometer results were only slightly more variable and equally failed to disclose any relation to the riboflavin intake.

TABLE 4

Average results of psychomotor tests, group B. "Tapping" is speed of tapping in contacts made in the first 10 seconds ("A") and in the third 10 seconds ("B"). "Pipe" is the ball and the pipe test, results in number of balls passed through the pipe in 60 seconds. "Maze" refers to the motor control tests; "A" gives the number of contacts per circuit and "B" is the total duration of those contacts in units of 1/120 seconds. F. F. F. is the flicker fusion frequency in flickers discriminated per second. Note that low scores are "good" in the maze test (A and B) and "poor" in all other tests. "After" refers to measurements made after 26 days of supplementation (11.2 mg. B₂ per day).

TEST	BEFORE	WEEKS ON LOW B ₂ REGIME										AFTER
		2	4	6	8	10	14	16	18	20	22	
Tapping A	68	70	69	70	69	72	72	72	72	74	74	74
Tapping B	58	60	60	60	61	59	63	62	62	63	64	63
Pipe	64	64	64	63	63	64	67	68	66	66	68	68
Maze A	86	83	74	72	77	77	71	61	66	56	55	56
Maze B	478	539	445	461	449	454	439	452	378	338	344	335
F. F. F.			61	61	60	60	60	60	63	62	62	61

Psychomotor results

The average results of the psychomotor tests with group B are given in table 4. Entirely similar results were obtained with group A and with the different subjects considered individually. There were some progressive training effects with the motor control (maze) test and smaller training changes in the pipe and tapping tests. These training

effects were entirely normal as judged by extensive studies on persons on other diets. The flicker fusion frequency, which has no appreciable training element, was not measured in the preliminary control period but all the measurements made were extraordinarily constant in each individual throughout the entire subsequent period.

Other measurements

There was a tendency for the hemoglobin concentration in the blood to rise slightly during the period of riboflavin restriction. This regressed on the return to a normal diet and was seen in both groups A and B (table 5). The interpretation of this is obscure.

TABLE 5

Average values for glucose tolerance, for blood hemoglobin and basal metabolism. Glucose in milligrams and hemoglobin in grams per 100 ml. of blood. B.M.R. as % of Mayo Clinic Standards.

GROUP PERIOD		GLUCOSE TOLERANCE				Hb	B.M.R.
		Basal	30 MIN.	60 MIN.	90 MIN.		
A	Before	69	107	111	81	15.3
A	84 Days	72	117	118	91	16.0
B	Before	76	124	99	92	15.7	-13%
B	84 Days	76	110	77	74	16.0	-15%
B	148 Days	75	100	83	86	15.8	-13%
B	After	78	121	87	89	15.5	-16%

The basal metabolism was remarkably constant at all times in group B (table 5); it was not measured in group A. All subjects at all times had B.M.R. values slightly below the "normal" averages of the Mayo Clinic but this is usual in this laboratory where the subjects are trained and more complete relaxation is customarily attained.

Average glucose tolerance results are given in table 5. The results differ somewhat in groups A and B but there was a distinct tendency for all the men in group B to exhibit a depressed tolerance curve during the period of riboflavin restriction and for this to return toward the original control level on the resumption of a normal diet. Again the significance, if any, of these changes cannot be surmised here. The basal blood sugar values were very constant throughout. The relatively low values reflect the method used which eliminates more non-glucose reducing substances than the more common clinical methods.

TABLE 6

Foods for representative days, together with riboflavin analyses and total calories. "W.K." is canned whole kernel corn; "lettuce, fr." is lettuce with french dressing. All cake, bread, pie, candy are made with special recipes using unenriched flour, little or no milk and minimal amounts of eggs. Cake and bread were fortified with purified casein and calcium phosphate and iron salts. "Cream, 25%" was prepared from 70% butter fat cream and salt solution.

DEC. 1, 1942	FEB. 2, 1943	FEB. 11, 1943
100 gm. Grapefruit juice	100 gm. Apple sauce	100 gm. Grapefruit
20 gm. Corn flakes	20 gm. Puffed rice	20 gm. Puffed rice
70 gm. Butter	80 gm. Butter	80 gm. Butter
100 gm. Cream, 25%	100 gm. Cream, 25%	100 gm. Cream, 25%
180 gm. Bread	240 gm. Bread	20 gm. Gran. sugar
20 gm. Gran. sugar	20 gm. Gran. sugar	180 gm. Bread
100 gm. Grape jelly	150 gm. Cranberry jelly	100 gm. Apple jelly
40 gm. Fudge	40 gm. Fudge	200 gm. Spaghetti
200 gm. Spaghetti with lettuce	30 gm. Hamburger	30 gm. Meat ball
25 gm. Bacon	50 gm. Fried onions	100 gm. W.K. corn
50 gm. Lettuce, fr.	150 gm. Potato salad	50 gm. Lettuce
100 gm. W.K. corn	50 gm. Lettuce	100 gm. Jello
100 gm. Gingerbread	100 gm. Peach preserve	50 gm. Cookies
100 gm. Hamburger	80 gm. Swiss steak	80 gm. Veal roast
150 gm. Baked potato	150 gm. Mashed potatoes	150 gm. Mashed potatoes
100 gm. Boiled onions	100 gm. Boiled cabbage	100 gm. Boiled carrots
50 gm. Raw carrots	100 gm. Chocolate cake	50 gm. Cranberry sauce
100 gm. Strawberry preserve		100 gm. Angel food cake
Final wet wt. = 1605 gm.	Final wet wt. = 1525 gm.	Total wet wt. = 1230 gm.
Total calories = 3090	Total calories = 3250	Total calories = 2810
Total B ₂ = 0.91 mg.	Total B ₂ = 0.92 mg.	Total B ₂ = 0.66 mg.

DISCUSSION

There have been relatively few prolonged studies on human beings maintained on known restricted intakes of riboflavin. Physical performance and other objective functional tests have not been reported before. In other respects it is useful to compare the present findings with those of Sebrell, et al. ('41) and the report of Williams, et al. ('43) which appeared just as the present research was being completed. In both of these studies the subjects were women inmates of institutions who were maintained at considerably lower caloric levels than our subjects. The Mayo Clinic subjects were particularly sedentary. If the riboflavin intake is considered in relation to total calories our subjects received about 50% more than Sebrell's subjects and about 12% less than those of Williams, et al. If total riboflavin per day is considered, then our subjects received about twice as much as Sebrell's subjects and almost 50% more than Williams' subjects. Further, our subjects went on the restricted regime from a college boarding house diet while

Sebrell's subjects may have been chronically somewhat malnourished and Williams' subjects were fortified by a prolonged high B₂ intake.

In any case the urinary excretions of riboflavin were of the same order of magnitude in all three studies. The average proportion of the dietary riboflavin which appeared in the urine was about 15% in Sebrell's subjects, 14% in those of Williams, and 12% in our subjects. The saturation test methods were not strictly comparable but it appears that our subjects showed somewhat lower apparent "saturation" than the Mayo Clinic subjects. The present results indicate that when the test dosage is of the same order of magnitude as the dietary intake and when the latter is low and fairly constant, then the proportion of total riboflavin intake which appears in the urine is relatively independent of the test dose. We agree with Najjar and Holt ('41) that the erratic results in the saturation tests reported by Axelrod, et al. ('41) may be explained by excessive test dosage.

Aside from urinary excretions no evidence whatever of riboflavin deficiency appeared in either our subjects or those of Williams, et al. Six out of the ten subjects in the series of Sebrell, et al., developed cheilosis in 89 to 232 days. The specificity of this lesion has been seriously challenged (e.g. Machella, '42; Machella and McDonald, '43) and we should note that the diet used by Sebrell, et al., may have been deficient in other B vitamins; pyridoxine has been reported to heal cheilosis in many cases (Smith and Martin, '40; Machella, op. cit.).

Estimation of the riboflavin requirement of man and the diagnosis of riboflavin deficiency are in a peculiarly unsatisfactory state. Youmans ('41) noted that the diagnosis of ariboflavinosis should be confirmed by objective changes induced by therapy with the vitamin. The situation has improved little in the succeeding 2 years. The extreme view that all capillary infiltration of the cornea indicates active or chronic riboflavin deficiency (Wiehl and Kruse, '41; Wiehl, '42; Borsook, et al., '43) appears to go far beyond the known facts. If we accept this theory then we should conclude that five out of six of our subjects suffered from slight riboflavin deficiency when they started the experiment. In any case neither the deficiency regime nor the "restoration" control period produced the slightest change in the eyes.

In the absence of other criteria urinary excretion and "saturation" tests have been used. Williams, et al. (op. cit.) state that their data show "unmistakable depletion of tissue stores of riboflavin" (p. 376), from which they conclude that the intake of 0.35 mg. per 1000 Cal. does not meet "requirements." Their data actually suggest that the tissue

stores of riboflavin in the first month or so of the restricted diet were larger than during the subsequent period and therefore indicate that a prolonged period of high riboflavin intake induces some temporary accumulation of the vitamin. Our results are in agreement. This does not necessarily mean that the body is then "depleted" in the sense of "starved" or "exhausted," when such an accumulation is reduced, nor does it necessarily follow that functional requirements of the body include such an excess store of riboflavin in the tissues. It is not proposed to discuss here the proper meaning of those popular terms "requirements" and "sub-clinical deficiency." The principal purpose of the riboflavin excretion measurements in the present study was to guarantee the estimate of the state of riboflavin nutrition. The results show that the riboflavin nutrition was as indicated by the diet and the food analyses. Both dietary and excretion data agree that the true intake of riboflavin was far lower than various "recommendations" and therefore did not meet requirements in this sense. But the main point of this work was to measure, by the most sensitive and objective means we could devise, the functional capacity of the body under these conditions. The results indicated that, within the limitations of time and subjects used, the sole disadvantage of the low intake which we found was in the small amount of riboflavin excreted.

The present results indicate that normal young men suffer no physiological or clinical handicap by restriction to an intake of riboflavin of 0.31 mg. per 1000 Cal. for a period of 5 months. Longer periods and very young or aged people may be another matter. However we are unaware of evidence on these points. Undoubtedly such a restricted diet does not provide a body reserve as large as would result from a greater intake.

Comparisons with animal experiments are of doubtful utility but we may note that dogs may fail to show symptoms for a long time on diets extremely low in riboflavin. Of the three dogs studied for about a year by Fraser, Topping and Isbell ('40), one developed symptoms in 7½ months, another some months later and the third never showed any abnormalities within the period of 11½ months of observation. The diet provided these dogs contained about 0.1 mg. of riboflavin per 1000 Cal.

SUMMARY

Studies were made under controlled conditions on six normal men students subsisting on a diet restricted in riboflavin but otherwise adequate. The restricted regime was preceded by 3 weeks of control

observations. Three of the men remained on the restricted intake for 84 days; the other three men were on the low regime for 152 days. The latter group was also studied during 12 weeks of "restoration" after the low regime. The diet actually eaten was directly analyzed for riboflavin. It averaged 0.99 mg. per day (3150 ± 100 Cal.) or 0.31 mg. per 1000 Cal.

Standardized tests covered 60 minutes of hard work on the treadmill, an anaerobic work test, glucose tolerance, tests of muscle power and several psycho-motor tests. Observations included pulse rates in rest, work and recovery, metabolism, blood lactate, glucose, pyruvate and hemoglobin, and urinary excretion of riboflavin with and without "saturation" test doses. Clinical examinations included slit lamp and other special observations. Observations and tests were made every other week except for the saturation tests, glucose tolerance and eye examinations, all of which were made about once a month. Analyses of urinary excretion were made each week.

The 24-hour urinary excretion of riboflavin averaged 12% of the dietary intake on the low regime and the recovery of saturation test doses of 1.0 mg. was very similar. After the first few weeks there were no progressive changes in either "regular" or "saturation" excretion.

All other results were essentially negative and constant. No clinical changes occurred. Work performance and the bodily responses to work were essentially unaffected by the dietary alterations.

It is concluded that normal young men suffer no physiological handicap from subsistence for at least 5 months on a diet providing 0.31 mg. of riboflavin per 1000 Cal. (0.99 mg. per day in balance at 3150 Cal.).

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THE INFLUENCE OF THE PROTEIN CONTENT OF THE DIET UPON FAT DIGESTIBILITY¹

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In the course of certain nutrition studies at this laboratory in which two levels of protein were being fed to rats, it was noticed that the groups receiving the lower protein diets excreted feces that were consistently light in color. Since no information was available which offered an explanation for this difference, measurements of fecal fat were considered advisable. Preliminary determinations showed that the fat content was considerably elevated in the feces of the rats receiving the lower protein diets. Therefore, a more critical study of fat digestibility was undertaken.

EXPERIMENTAL

Two groups of adult rats (approximately 200 days of age) that had been raised from the time of weaning on semi-purified diets were kept in large-mesh wire bottom cages that were elevated several inches above a metal tray covered with heavy paper. The food was placed in small glazed clay feeders and the rats were allowed to eat ad libitum. Food intake was measured over 3- and 4-day periods. Spillage was noted, but was in no case serious enough to warrant corrections. When changes in the type of fat were made the new diets were fed for 1 week prior to starting collections. Feces were collected for the same periods and dried in an oven at 110°C. The fat was extracted with low boiling petroleum ether after saponification, acidification with hydrochloric acid and dilution with water. In calculating the fat digestibility no correction was made for endogenous fat excretion.

The diets contained either 12 or 28% casein, 5% yeast, 4% salt mixture [a modification of the mixture of Hubbell, Mendel and Wakeman ('37) as described by Clausen, Barnes and Burr ('43)], 18% of the test fat, and sucrose to make up the balance. Three drops of a mixture

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of 2 parts cod liver oil and 1 part wheat germ oil were administered by mouth with an eye dropper twice weekly. All diets were kept in 1-gallon glass jars and stored in a refrigerator. The fats studied were a steam rendered lard (melting point 33–35°C.); a specially stabilized lard containing 8 to 12% lard which had been hydrogenated to an iodine value of approximately 30 (melting point 39–41°C.); a commercially packaged butter which was purchased during the winter months (melting point of the butterfat 26–28°C.); and an experimentally developed butter from cows which had been fed a diet known to cause an increase in the hardness of the butterfat and which in addition contained 5% completely hydrogenated vegetable fat. The melting point of the fat from this special butter spread was 39–41°C. Additional constants of the test fats are shown in table 1. Melting points were measured with

TABLE 1
Constants for the different fats.

TYPE OF FAT	IODINE VALUE (WIJS)	THIOCYANOGEN VALUE	SAPONIFICATION VALUE	REICHERT MEISSEL NUMBER
Steam lard	65.50	56.02	194.0
Special lard	60.64	50.93	194.0
Standard butter	32.08	35.92	224.0	33.42
Special butter spread	39.40	33.50	213.0	20.93

a special melting point determination "hot-plate". Iodine, thiocyanogen, saponification and Reichert Meissl values were determined by the methods of the American Oil Chemists' Society ('41). Four groups of male and four groups of female rats were used in this study. They had been raised from the time of weaning on either the lower or higher protein diets containing either the specially stabilized lard or butterfat from the commercially packaged butter. Growth was normal as judged by comparison with stock animals, but the rats receiving the higher protein diet grew at a slightly more rapid rate. After making digestibility studies on these groups the rats receiving the special lard were fed a diet containing steam rendered lard. The rats which had been receiving butterfat were given diets containing the special butter-spread. One week after making these changes fecal collections were started.

RESULTS

The digestibility data are shown in table 2. In every case the rats receiving the lower protein diet had an increased amount of fecal fat. Perhaps this does not offer a complete explanation for the very light color of the feces from these rats, but it does indicate that the fat content

TABLE 2
Fat digestibility and protein content of the diet.

GROUP NO.	TYPE OF FAT	COLLECTION NO. OF BAYS	FAT INTAKE ¹	WEIGHT OF FECS ¹	FECAL FAT	FAT ABSORBED ¹	DIGEST ¹ BILITY ²	NO. OF BAYS	FAT INTAKE ¹	WEIGHT OF FECS ¹	FECAL FAT	FAT ABSORBED ¹	DIGEST ¹ BILITY ²
F E M A L E S													
			gm.	gm.	%	gm.	%		gm.	gm.	%	gm.	%
1. Low protein	Steam lard	1	2.12	0.68	5.14	2.08	98.0	6	2.34	0.81	7.47	2.28	97.5
		2	1.88	0.54	4.91	1.85	98.4	6	2.05	0.86	7.25	2.00	97.5
		3	1.30	0.46	5.81	1.27	97.6	6	1.89	0.59	4.85	1.85	97.5
Average		7	1.77	0.56	5.28	1.73	98.0		2.09	0.75	6.52	2.04	97.6
2. High protein	Steam lard	1	2.34	0.94	0.35	2.33	99.9	6	2.50	0.88	2.57	2.47	98.8
		2	1.51	0.59	0.29	1.50	99.9	6	2.27	0.89	2.40	2.25	99.1
		3	1.51	0.69	2.06	1.79	99.5	6	2.23	0.92	5.79	2.18	97.8
		4	1.80	0.69	3.33	1.78	98.9	6	2.57	0.92	5.50	2.52	98.0
Average		5	1.86	0.73	1.51	1.85	99.5		2.39	0.90	4.06	2.35	98.4
3. Low protein	Special lard	1	1.71	0.52	22.90	1.59	93.1	6	1.91	0.52	24.46	1.78	93.4
		2	1.49	0.60	15.85	1.40	94.0	6	1.46	0.75	12.59	1.35	92.6
		3	2.36	0.57	19.27	2.25	95.4	6	0.95	0.82	14.51	0.83	87.4
		4	1.71	0.61	18.43	1.60	93.6	6	2.05	0.78	15.39	1.93	94.2
Average		7	1.82	0.57	19.11	1.71	94.0		1.59	0.72	16.74	1.47	91.9
4. High protein	Special lard	1	1.57	0.61	12.50	1.49	94.7	7	2.14	0.87	15.10	2.01	94.0
		2	1.37	0.60	7.91	1.32	96.4	7	1.89	0.91	9.32	1.81	95.7
		3	1.03	0.66	3.52	1.01	98.1	7	2.23	1.02	10.40	2.12	95.1
		4	1.49	0.71	7.61	1.44	96.7	7	2.04	0.99	6.00	1.98	97.0
Average		6	1.36	0.64	7.83	1.31	96.7		2.07	0.95	10.20	1.93	95.4
5. Low Standard butter		1	1.04	0.53	23.14	0.92	88.5	4	0.97	0.62	23.98	0.79	81.5
		2	2.11	0.63	23.51	1.96	93.0	4	1.95	0.75	18.98	1.81	92.9
		3	1.74	0.83	10.92	1.65	94.8	4	1.80	1.27	13.81	2.31	93.6
Average		6	1.63	0.66	13.86	1.51	92.1		2.40	0.86	20.26	1.64	89.3
6. High Standard butter		1	1.50	0.51	7.45	1.46	97.3	7	1.73	0.76	9.22	1.66	96.0
		2	1.55	0.60	4.46	1.52	98.1	7	2.20	0.78	7.38	2.14	97.4
		3	1.65	0.62	8.21	1.60	97.1	7	1.64	0.80	12.59	1.54	94.0
		4	1.74	0.60	4.23	1.71	98.2	7	1.98	0.87	8.44	1.91	96.5
Average		5	1.61	0.58	6.09	1.57	97.7		1.89	0.80	9.41	1.81	96.9
7. Low protein	Special butter	1	2.43	0.92	10.49	2.28	93.9	4	2.59	1.09	16.24	2.41	93.0
		2	1.73	0.67	16.05	1.62	93.8	4	2.07	0.90	19.04	1.90	91.7
		3	1.85	0.71	23.25	1.69	91.5	4	2.41	1.02	12.66	2.28	94.6
		4	1.98	0.66	28.10	1.79	90.5	4	2.45	1.06	22.50	2.21	90.2
Average		6	2.00	0.74	19.47	1.84	92.4		2.38	1.02	17.61	2.20	92.4
8. High protein	Special butter	1	2.17	0.85	15.88	2.04	94.1	7	2.41	0.74	18.63	2.27	94.1
		2	1.65	0.62	5.04	1.62	98.3	7	1.98	0.89	8.71	1.90	96.0
		3	1.64	0.70	13.20	1.55	94.6	7	2.41	1.00	6.34	2.35	97.5
		4	1.99	0.71	8.56	1.93	97.1	7	2.52	1.04	7.95	2.44	97.0
Average		5	1.86	0.72	10.67	1.78	96.0		2.23	0.92	10.41	2.24	96.2

¹ Calculated as per rat per day. By "fat absorbed" is meant the difference between fat intake and fecal output.
² Not corrected for endogenous fat excretion.

was partially responsible. In addition, when fat intake is taken into account, digestibility (as calculated from the difference between intake and excretion) of all of the fats was decreased in the lower protein groups. This decrease was least serious with the best digested fat (steam lard) and was of greater magnitude with the other fats which exhibited poorer digestibilities. Another observation of importance is the effect of incorporating hydrogenated fats into lard and butter. Calculations of the fatty acid composition of the steam rendered and special stabilized lards gave, respectively: saturated acids, 34.5 and 40.4%; oleic acid, 54.6 and 48.2%; and linoleic acid, 11.0 and 11.4%. This indicates that the special lard contained approximately 6% more saturated fatty acids than the steam rendered lard. Five per cent hydrogenated vegetable fat (iodine value 10) was added to the special butter spread. There was no apparent alteration in the digestibility of the "hardened" butterfat while a marked decrease was observed with the "hardened" lard, especially in the high protein groups.

DISCUSSION

Munk and Rosenheim (von Noorden, '07) have conducted studies in which dogs were fed very low protein diets for 6 or 8 weeks and it was found that digestive powers were seriously upset, finally resulting in death. Fat absorption was most seriously impaired, but the presence of numerous, severe lesions of the intestinal tract led to the conclusion that impaired intestinal functions were the cause of the faulty absorption. Magnus-Levy (von Noorden, '07) quotes studies by Jägerroos and by Chittenden in which no sign of decreased fat digestibility was evident in experimental animals and humans which were maintained on low protein diets. Coffey, Mann and Bollman ('40) measured fat excretion by dogs fed different types of basal diets and found only a slight variation (range 2.11 to 3.99% of the ingested fat excreted in the feces) in the percentage of fecal loss of fat; their high protein diet gave the lowest digestibility, but in this study the fat content of the diets was not constant.

In the experiments reported here the rats were maintained from the time of weaning on diets containing the specified amounts of protein. They were all in good condition and did not exhibit any gross evidence of intestinal pathology. The effects noted were probably not due to lesions of the intestinal tract, but post mortem examinations have not been performed. It is possible that the decreased fat absorption was induced through long continued feeding of the experimental diets and would not be observed in short time studies. The explanation for the

decreased absorption remains obscure. The possibility of a carbohydrate as well as a protein influence upon fat absorption must be given some consideration for decreases in the protein content of the diets were compensated for by corresponding increases in sucrose. An inhibition of fat absorption brought about by increasing the carbohydrate content of the diet does not appear logical, but Briggs, Heller and Wall ('40) have noted that the inclusion of molasses in an oat or corn-alfalfa ration for lambs lowered fat digestibility by 17-18%.

Magnus Levy (von Noorden, '07, p. 55) has presented evidence of an enhanced digestibility with increases in the amount of fat in the diet. Hoagland and Snider ('43), working with rats, have found this to be true for certain hard fats of low digestibility but not for highly digestible oils. In the present communication evidence is given that another dietary ingredient influences fat digestibility. Both the fat and protein contents of the basal diet may remain well within normal nutritional limits and yet exert definite effects upon fat digestibility. Certainly if digestibility measurements from different laboratories are to be compared, a standardized diet must be employed.

The comparisons of digestibilities of lard and butter to which hydrogenated fat has been added are of interest in connection with the use of specially developed "hard fats" which will remain solid at elevated temperatures. The increased fecal loss shown by the blended lard is to be expected if hydrogenation produced much stearic acid. More difficult to understand is the lack of effect when hydrogenated fat was added to butter. The fact that normal butterfat has a relatively low digestibility may have some bearing. One other possibility is that the unique fatty acids in butterfat in some manner counteract the poor digestive characteristics of the hydrogenated fat.

SUMMARY

Comparisons of fat digestibilities in rats receiving diets containing approximately 14 or 30% protein have shown that the lower protein intake is associated with a lower fat digestibility. A well absorbed fat such as lard is influenced only slightly while more poorly digestible fats such as lard containing hydrogenated fat and butterfat are more seriously affected.

The differences in digestibility that are brought about by varying the protein intake emphasize the importance of standardizing the basal diet for all measurements of fat digestibility.

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A STUDY OF THE DIET OF TWENTY WOMEN IN A MODERATE-INCOME GROUP¹

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A previous study by the above authors ('43) of the diet of women in a low-income population group has been reported. This study was made by assaying food samples which duplicated in kind and amount the food actually consumed during a day. In the present study this same method was used for a moderate-income group in order to obtain supplemental data on the nutritive value of food as actually consumed. It was necessary to terminate the study in February when food rationing was instituted so that it is not as extensive as was planned. Before this time diet samples had been collected from twenty women, twelve of whom were young married women with children. These twelve women made up a group comparable to the low-income mothers on whom we previously reported. Of the remaining eight women, four were members of the faculty of the Department of Home Economics and four were advanced students in this department.

EXPERIMENTAL

Selection of subjects and collection of samples

The group of twelve young married women who collected diet samples were, with two exceptions, wives of University professors. They were selected because of their interest in the study and willingness to cooperate. The ages of this group ranged from the late twenties to the early forties, the majority falling between 32 and 37 years. All of these women were considered moderately active. The average number of children per mother was 2. One woman was pregnant; none were lactating. Definite information on income was not obtained, but incomes probably ranged from \$2500 to \$5000 per year. Instructions for the collection of the samples were the same as were given the low-income group, and the aim as before was the duplication in

¹This research was aided by a grant from the Williams-Waterman Fund.

quantity and kind of the food consumed daily by the individual. The faculty members and students in the Department of Home Economics were asked to collect diet samples because their understanding of and interest in the project guaranteed the utmost care in collection. It was thought desirable to have a group in which accuracy in this respect was beyond question.

Methods of assay

These were the same as were used in the previous investigation and are described in the report of this study. Assay for thiamine was carried out in the fermentometer following the directions of Frey, Atkin, and Schultz ('42). Microbiological methods were used for the determination of riboflavin, pantothenic acid, and niacin. These are fully described in the University of Texas monograph entitled, "Studies on the Vitamin Content of Tissues, I" ('41). Calcium was determined according to the A. O. A. C. method. The colorimetric method of Koenig and Johnson ('42) was used for phosphorus. In order to check the figure of 5 calories per gram of dry sample which was obtained in the low-income study by bomb calorimeter determinations, another series of these determinations were run. Twenty samples were so treated, and the results showed a variation from 4.875 to 5.4 calories per gram and an average value of 5.1. The value of 5 calories used in the low-income study was confirmed as sufficiently accurate for caloric estimations.

RESULTS

Table 1 presents the data obtained from assay of the diets of the group of young married women (group I). These data are comparable with the results obtained on the low-income group since the groups are similar in age and size of family and differ in income, education, and intelligence. The four women faculty members made up group II, and the results obtained on their diets are given in table 2, as are also the results of the analysis of the diets of the four students that make up group III.

DISCUSSION OF RESULTS

Calorie intakes

In the previous study, it was found that the calorie intakes of women in the low-income group were startlingly low, ranging from 620 to 2050 calories per day and averaging 1145. These women were classed as sedentary on the basis of their observed activity. The average intake was about half the 2100 calorie allowance for sedentary women,

TABLE 1

Nutritive value of diets of women on moderate income level (group I) and comparison with similar values for low-income level.

SUBJECTS	ENERGY	PROTEIN	CALCIUM	PHOS- PHORUS	RIBO- FLAVIN	PANTOTHENIC ACID	NIACIN	THIAMINE
	<i>Calories per day</i>	<i>g./day</i>	<i>g./day</i>	<i>g./day</i>	<i>mg./day</i>	<i>mg./day</i>	<i>mg./day</i>	<i>mg./day</i>
M ₁	1865	74.2	1.09	1.54	3.05	7.25	10.34	.65
C ₁	121571	1.18	3.80	5.51	.41
L	1700	1.07	1.45	4.21	7.35	.75
C ₂	1985	53.4	.98	.99	2.09	6.47	9.87	1.04
M ₂	2445	79.9	1.63	1.74	2.73	8.48	9.07	1.16
B ₁	2120	73.5	1.29	1.09	1.72	3.90	9.65	1.14
P	1885	74.6	1.26	.80	1.89	3.97	10.00	1.11
H ₁	1570	59.7	.65	.61	1.06	2.81	9.62	.82
H ₂	1405	54.6	.96	.84	1.65	6.22	11.44	.76
B ₂	1095	38.4	.40	.59	.96	2.93	6.68	.25
F	1195	40.3	.53	.59	1.24	4.14	9.62	.55
K	1530	49.4	.47	.58	1.55	3.41	9.70	.44
Average	1667	59.8	.93	.93	1.71	4.79	9.07	.72
Low-income Group	1145	33.8	.38	.72	.78	2.41	4.13	.49
Percentage Increase	45%	77%	145%	30%	120%	99%	120%	47%

TABLE 2

*Nutritive value of diets of four faculty women (group II)
and four University students (group III)*

SUBJECT	ENERGY	PROTEIN	CALCIUM	PHOS- PHORUS	RIBO- FLAVIN	PANTOTHENIC ACID	NIACIN	THIAMINE
	<i>Calories per day</i>	<i>g./day</i>	<i>g./day</i>	<i>g./day</i>	<i>mg./day</i>	<i>mg./day</i>	<i>mg./day</i>	<i>mg./day</i>
Group II								
L	1900	61.9	1.17	1.19	1.68	6.65	9.02	1.00
W	1605	53.3	.73	.89	1.88	5.92	10.71	1.00
S	1595	58.8	.76	.86	2.56	6.15	8.31	1.08
T	1680	57.1	.98	.84	1.61	4.49	6.78	.71
Average	1720	57.8	.91	.94	1.93	5.80	8.71	.95
Group III								
F	2020	79.4	...	1.14	2.49	5.37	9.60	.86
M	1900	69.0	.93	.91	1.91	4.61	10.79	.98
H ¹	1970	67.4	.90	1.20	2.97	7.40	8.79	1.10
H ²	1795	60.1	1.07	1.01	2.53	5.45	8.80	1.12
Average	1920	69.0	.96	1.07	2.47	5.71	9.49	1.01

recommended by the Committee on Foods and Nutrition, National Research Council. The present moderate-income group of women (group I, comparable to the low-income group of last year) had an average calorie intake of 1667 with a range of 1095 to 2445. While the average intake is 45% above that of the low-income groups, it is still far below the recommended allowance. Most of the women in group I should, in the opinion of the investigators, be classed as moderately active, and, if so classified, the average intake is 66% of the recommended allowance of 2500 calories. It should also be pointed out that, in group I, only two of the women had intakes above the allowance recommended for a sedentary woman and only one approached that for a moderately active woman.

The intake of the women in group II averaged 1720 calories per day, and was slightly higher than that of group I, but the group of students (group III) had a considerably higher average intake, 1920 calories. In no case, however, does the average intake closely approach the recommended allowance for a sedentary woman, i. e., 2100 calories.

There was a lack of correlation between body weight and calorie intake in group I. The two women with the two lowest intakes were inclined to overweight while the one with the highest intake was extremely thin. The other members of group I and all of the women in groups II and III were in the normal range of weight for height.

Basal metabolism determinations were made for thirteen of the twenty women comprising the three groups. The results are given in table 3, together with the calorie intake and the percentage deviation of total calories from the basal. Two of the women had intakes slightly below basal requirements. The percentage increase over the basal varied from 6 to 81%. While all of the basals were within a normal range, all were on the minus side, determinations showing a range of -5 to -15%. A long series of basal determinations made through a period of years on presumably normal students in the University showed an average variation of -8%. The basal metabolism of the women tested was considered not unduly low for this part of the country, although it is possible that there is some correlation between the low basals and the low calorie intakes.

In a dietary study made by Youmans ('42) on a low-income group, as many as a third of the group had calorie intakes that were less than 30% above basal requirements. The same proportion holds true for the present group.

As a result of the study of the low-income group the possibility that the recommended allowances for calories had been placed too high was suggested, and the lack of correlation between calorie intake and body weight was pointed out. The results obtained on the moderate-income group of women emphasize this possibility and indicate again a lack of correlation between body weight and calorie intake.

TABLE 3

Basal calorie requirements, actual calorie intakes, and percentage deviations.

SUBJECT	BASAL CALORIE REQUIREMENTS	CALORIE INTAKE	PERCENTAGE DEVIATION
Group I			
C ₁	1099	1215	+ 11
L	1362	1700	+ 26
M ₁	1351	2445	+ 81
P	1230	1885	+ 53
H ₁	1319	1405	+ 6
B ₁	1119	1095	- 2
F	1356	1195	- 13
Group II			
L	1285	1900	+ 48
W	1111	1605	+ 43
S	1216	1595	+ 31
T	1285	1680	+ 31
Group III			
F	1168	2020	+ 73
M	1298	1900	+ 54

Protein intakes

The average protein intakes for groups I and II were practically the same and were very close to the allowance recommended for women, i. e., 60 gm. The protein intake of the student group was somewhat higher, as would be expected from the higher calorie intake. The average protein intake of group I was 77% higher than for the comparable low-income group. Samples were not available for two of the twenty women studied, but, of the eighteen determinations made, only three were more than 10% below the 60-gm recommended allowance.

Mineral intakes

It is interesting that in all three of the groups of women studied this year the calcium and phosphorus intakes were nearly the same

and were approximately 1 gm. each. This contrasts markedly with the results obtained on the low-income group for which an average intake of 0.38 gm. of calcium and 0.72 gm. of phosphorus was indicated. Undoubtedly much larger amounts of milk and green, leafy vegetables were consumed by the higher income group, and it is probably the difference in the consumption of these two foods that accounts for the difference in mineral consumption of these two groups. Phosphorus is not included in the new recommended allowances, but the old "standard allowance" of 1.32 gm. per day per person is approximately one-third more than was furnished by the diets of the present group and two-thirds more than was indicated for the low-income diets. It is difficult to account for the low phosphorus content of these diets, especially in view of the high calcium intake for the moderate-income group. Usually the phosphorus content of a diet is higher than the calcium content, but this was not true for the present group.

Vitamin intakes

As in the previous study assays were made for four of the vitamins: thiamine, riboflavin, niacin, and pantothenic acid. The results of the vitamin assays for group I, together with the corresponding figures for the low-income group are given in table 1. Pantothenic acid intake was twice that indicated for the low-income group, while riboflavin and niacin intakes were more than doubled; thiamine intake, however, was only 47% greater. The better choice of food by the higher-income group is shown by the fact that, while the calorie intake was increased only 45% over that of the low-income group, the intake of all other nutrients, with the exception of phosphorus and thiamine is very much greater than 45%. Figures for the student and faculty groups (tables 2 and 3) show 20-30% larger intakes than for group I of all vitamins except niacin. The intake of niacin varied only slightly for the three groups.

Using the recommended allowances for a moderately active woman for comparative purposes, it may be shown that the average riboflavin intake for group I was 80% of the recommended allowance of 2.2 mg., that for group II, 90% and that for group III, 112%. Similar figures for niacin (recommended allowance, 15 mg.) are 60%, 58% and 63%; for pantothenic acid (using the estimate of 10 mg. suggested by R. J. Williams ('41) 48%, 58%, and 57%; for thiamine (recommended allowance, 1.5 mg.) 48%, 63%, and 70%. Only in the case of riboflavin is a satisfactory intake indicated. This was achieved by a large

milk consumption. As another illustration of the more nearly satisfactory intake of riboflavin it may be pointed out that while 50% of the twenty women studied have riboflavin intakes above 1.8 mg. (recommended allowance for a sedentary woman) none of the women have intakes of thiamine or niacin equal to the allowance for a sedentary woman. It was found, for the low-income group, that the intakes of niacin, thiamine and riboflavin were approximately one-third of the allowance for a sedentary woman while the intake of pantothenic acid was about one-fourth of the suggested 10 mg. standard.

SUMMARY

In estimating the deficiencies of the diet of a group of women on a moderate income, the recommended allowances for a moderately active woman have been used. For a similar purpose, but for a low-income group, the allowances for a sedentary woman were employed. This is in accordance with the opinion of the investigators concerning the activity of the subjects.

The general conclusions in regard to the low-income group were that calorie intakes were from one-half to three-fourths of the recommended allowance; that the average intakes of thiamine, niacin, and riboflavin were approximately one-third and the intakes of protein, calcium, and phosphorus approximately one-half of the allowance recommended. Pantothenic acid was only about one-fourth of the 10 mg. suggested by R. J. Williams ('41) as representing adequacy for this vitamin. Similar conclusions for a comparable group of women on a moderate income level, were that calorie intakes varied, on the whole, from one-half to four-fifths of the allowance recommended, and that the average intakes of protein and calcium indicated no deficiency in these nutrients. A slightly deficient intake of phosphorus is apparent. The average intake of riboflavin was four-fifths, that of niacin three-fifths, and that of thiamine one-half of the allowances recommended for these vitamins. Pantothenic acid intake was a little less than half the amount suggested as representing adequacy. If the allowances for a sedentary woman are used, for the moderate-income group, the data indicate 80% adequacy in calories, 95% in riboflavin, 75% in niacin, and 68% in thiamine. The increase in calorie intake in the moderate-income group was small in comparison with the increase of the other nutrients with the exception of phosphorus. The reasons for believing that the calorie intakes obtained in the present study emphasize the possibility of a too generous recommended allowance have already been discussed.

It was not possible to determine the nutritional status of the subjects of this study as no one competent to make the necessary physical examinations was available.

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STUDIES ON VITAMIN E DEFICIENCY IN CHICKS¹

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FIVE FIGURES

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The two main symptoms of vitamin E deficiency in chicks are exudative diathesis and encephalomalacia. They may appear separately or concomitantly depending upon the composition of the diet. The experiments reported here were designed to examine the manner in which modification of various components of vitamin E deficient diets may influence these symptoms, in the hope that such observations might throw additional light upon the physiological rôle of vitamin E.

In order to evaluate the results of these studies it seems necessary to outline the chief characteristics of these two symptoms and the existing state of our knowledge concerning them.

Exudative diathesis, a condition in which plasma exudes from the capillaries, appears as massive accumulations of fluid in the subcutaneous tissue or as edema of muscles and connective tissue more generally (Dam and Glavind, '38).² The affected tissue, especially the adipose tissue, is usually reddish due to fine diffuse hemorrhage. Microscopically there is edema, slight extravasation of red cells and migration of white cells in the affected tissue, and a mild eosinophilia (Dam and Glavind, '39b). The extravasated fluid, which is often greenish from decomposition products of hemoglobin, has approximately the same composition as that of normal plasma. An augmented capacity of the tissue to take up trypan blue intravenously injected (Dam and Glavind, '40) suggests an increased permeability of the capillaries. Since the specific

¹ Aided by a grant from the Josiah Macy, Jr. Foundation.

² The fact that vitamin E is the factor which protects against the exudative diathesis has been well established (Dam and Glavind, '39a, '39b; Bird and Culton, '40; Dam, Glavind, Prange and Ottesen, '41). A level of 10 mg. of d, l- α -tocopherol acetate mixed with 100 gm. of the diet always affords sufficient protection. β -tocopherol is much less active against this disease than α -tocopherol. During the study of the distribution of the anti-exudate factor in foods it was found that the lipid of rose hips (*Rosa rugosa*) is approximately ten times as rich in vitamin E as wheat germ oil (Dam, Glavind, Prange and Ottesen, '41).

gravity of the serum is normal³ it seems unlikely that a decrease in serum proteins contributes to the appearance of the exudates. Plasma prothrombin and the thromoplastin in the affected tissue are normal. There is no decrease in osmotic resistance of the red cells, no increase in sedimentation rate, and no elevation of body temperature (Dam, unpublished studies).

Although individual chicks of an experimental group as well as different shipments of chicks may vary somewhat with respect to the appearance and severity of the symptoms, the basal diet 182 (table 1) used in most of the present studies usually produces exudates within 14 to 30 days in chicks 1 day old at the beginning of feeding. The exudates generally appear first in adipose tissue, later affect muscles, and are rare in the pericardial and peritoneal cavities. They may disappear spontaneously and recur later in the same chick with no change of the dietary procedure. Absorbed exudates usually leave a buff coloration in the adipose tissue, and sometimes leave a necrotic degeneration of breast and leg muscles leading to permanent invalidism. Severe exudates may be associated with deep and forced respiration. Encephalomalacia occurs occasionally. Chicks grow somewhat subnormally on this diet, attaining weights of 120 to 160 gm. in 4 weeks.

Chicks showing spontaneous disappearance of the symptoms and continued for longer periods on the diet often die with a perforating ulcer of the gizzard, which is unrelated to vitamin E and can be prevented by other modification of the diet.

Using a somewhat different type of diet (table 1), Bird and Culton ('40) report the exudative diathesis to be associated with a considerable accumulation of fluid in the pericardium and peritoneal cavity, indicating that the degree and localization of the exudation may be influenced by the makeup of the diet. The addition of 5% cod liver oil to the Bird and Culton diet has produced, in the author's laboratory, essentially the same symptoms as observed when chicks were fed the exudate-producing diet 182.

Encephalomalacia, the other symptom, was first observed by Pappenheimer and Goettsch ('31).⁴ Chicks reared on their diet 108 (table 1)

³ Fasting values of 1.0154, with a standard deviation of 0.0006, for five 40-day-old chicks receiving the exudate-producing diet, compared with 1.0157, with a standard deviation of 0.00015, for ten 34-day-old chicks on a commercial chicken diet.

⁴ The evidence for the relation of encephalomalacia to vitamin E deficiency rests on the finding of a protective factor in the non-saponifiable fraction of various lipids of vegetable origin (Goettsch and Pappenheimer, '36). When synthetic d, l- α -tocopherol became available this substance was found to protect (Dam, Glavind, Bernth and Hagens, '38; Pappenheimer, Goettsch and Jungheer, '39).

become atactic, may fall when trying to walk, get cramps and carry out uncoordinated movements with legs, wings and neck. Frequently they lie on the side with legs stretched or assume a sitting position with the back supported against the wall of the cage. Upon touching, violent movements with legs ("bicycling") and wings occur. In milder cases the birds are only slightly atactic or sit with the body raised and slightly

TABLE 1

	BASAL DIET NO. 32 ¹	BASAL DIET NO. 182 ¹	BIRD AND CULTON'S DIET ⁷	PAPPEN- HAIMER AND GOETTSCH DIET 108 ⁹	BASAL DIET NO. 190
Casein, unextracted	22.8	...
Casein, alcohol-extracted	15	15	15
Skimmed milk powder	54	16.7	...
Dried yeast, unextracted	5.6	...
Dried yeast, ether-extracted	10 ²	10 ²	10 ²
Corn starch, unextracted	22.2	...
Corn starch, alcohol-extracted	24.6
Sucrose	59.8	54.6	44
Gelatine	8	8
Gum arabic	5	5	5
Salt mixture	2 ³	7.2 ⁴	1.1332 ⁵	7.2 ¹⁰	7.2 ⁴
Ground limestone	1
L-cystine	0.1	0.1	0.1
Choline chloride	0.1	0.1	0.1
Lard	23.3 ⁴	30 ⁴
Cod liver oil	5 ⁴	5 ⁴	fed 3 X weekly	2.2 ⁴	...
Vitamin K substitute	1 mg. ⁶	1 mg. ⁶	1 mg. ⁵
Vitamin A, D concentrate	1 drop 2 X weekly ¹¹

¹ This diet is deficient in the peptic ulcer factor (cf. text).

² Fleischmann type 2019. Ether-extraction hastens the onset of the symptoms considerably when this type of yeast is used. The Carlsberg yeast used in Copenhagen apparently needed no ether-extraction.

³ Salt mixture no. 3. 2 gm. consists of: CaCO₃ 1457.8 mg., MgCO₃ 72.89 mg., NaCl 320.5 mg., Ferric citrate 116.5 mg., Diiodotyrosine 0.018 mg., CuSO₄ 5H₂O 10.92 mg., MnSO₄ 4H₂O 21.84 mg.

⁴ Added fresh to the diet every day.

⁵ 2-methyl-1, 4-naphthohydroquinone diacetate or the tetra-sodium-salt of 2-methyl-1, 4-naphthohydroquinone-diphosphoric acid ("Synkavite, Roche").

⁶ Consists of 7.16 gm. of McCollum's salt mixture no. 185, 0.966 mg. KI, 9.93 mg. CuSO₄ 5H₂O and 39.8 mg. MnSO₄ 4H₂O.

⁷ This diet should be supplemented with vitamin K.

⁸ Consists of NaCl 1 gm., MnSO₄ 4H₂O 0.012 gm. and CuSO₄ 5H₂O 0.0012 gm.

⁹ This diet should be supplemented with vitamin K and MnSO₄. It gives a marked perosis when fed without added manganese. In the formula given in table 1 the roughage (10% paper pulp) has been omitted, because it has no influence on the experiments.

¹⁰ McCollum's salt mixture no. 185.

¹¹ Consists of: vitamin A concentrate from fish liver oil (containing 10⁶ units per gram) 0.300 gm., vitamin D concentrate (Delsterol, containing 200,000 chick units per gram) 0.200 gm., oleic acid 24,500 gm. The concentrates were obtained from Distillation Products, Inc., Rochester, N. Y. 1 drop (29 mg.) represents 350 units A and 46 chick units D.

swaying. Forced breathing may be apparent. Localized or diffuse areas of edema, fine hemorrhages, and necrosis are usually seen in the cerebellum or the cerebrum. The extent or magnitude of these lesions is not always related to the severity of the symptoms observed. According to Pappenheimer and Goettsch ('31) the cerebellar lesions are characterized histologically by edema, with disorganization of the fibrillar and cellular elements, degeneration of the Purkinje cells and those of the granular layer, small hemorrhages scattered in both white and gray matter, and hyaline thrombosis of capillaries in and around the necrotic areas. The authors stress the capillary thrombosis as the primary cause of the necrotic lesions.

As in the case of exudates, spontaneous recovery with or without recurrent attacks, a buff coloration persisting for some time at the site of earlier lesions, and subnormal body growth are common phenomena in chicks fed the encephalomalacia-producing diet 108; while exudative diathesis rarely appears and no peptic ulcers occur. The water content of the affected parts of the brain is increased (Pappenheimer, Goettsch and Jungherr, '39). A reported decrease in cholesterol content (Adamstone, '41) must be taken with some reservation since the values given for normal chick brain are considerably lower than those generally recorded for brain tissue.

METHODS OF RECORDING SYMPTOMS

One-day-old white leghorn chicks, ten in each experimental and in each control group, were reared in ordinary brooders with raised screen floors and inspected daily, including Sunday, for the appearance of symptoms. The latter are recorded in the graphs as follows. Abscissae indicate age in days, ordinates the number of animals showing symptoms within a given number of days; giving one stair-case curve for exudate (full line) and another for encephalomalacia (broken line), each beginning from the bottom line. In order to give a more accurate representation of the development of the symptoms, exudates are graded by recording a reddishness of the fat tissue which is visible through the skin but not yet accompanied by a palpable exudate as " $\frac{1}{2}$ exudate", and adding another " $\frac{1}{2}$ exudate" for the same chick when a palpable exudate appears. One animal can be represented by only 1 unit on the ordinate since it is only the beginning of the symptom (eventually divided in two stages) and not its duration or recurrence which is recorded. Animals dying without showing exudate or encephalomalacia are recorded by full lines and broken lines, respectively, moved

down 1 unit from the top (or $\frac{1}{2}$ unit if the animal has had " $\frac{1}{2}$ exudate"). The end of each experiment is indicated by a black triangle. In only a few instances were symptoms found at autopsy which could not be observed in vivo. Experiments carried out simultaneously are grouped together in one series. Weight charts are not given but in cases where changes of the diet caused marked changes in body weight specific mention of this effect is made in the text, since one might expect that slow growth would retard the symptoms and rapid growth accelerate them.

FACTORS AFFECTING THE APPEARANCE OF EXUDATES

The salt mixture

The earlier experiments on exudative diathesis (Dam and Glavind, '38, '39a, '39b) were carried out with a lower salt content than that indicated in diet 182; namely, 2% of salt mixture no. 3 (cf. diet 32, table 1), which only approximately supplements the inorganic constituents of 10% yeast and 15% casein. As shown in series 1, the symptoms came much later with such a diet (exp. 3) than with the basal diet 182 containing 7.2% of McCollum's salt mixture no. 185 supplemented with Cu and Mn (exp. 1) or after the addition of sufficient NaCl to diet 32 to give the mineral constituents about the same osmotic concentration as in diet 182 (exp. 4; see also Dam and Glavind, '42).⁵ The accelerating effect of NaCl, also observed by Bird ('43), is in agreement with the common clinical experience that salts which tend to enter the extracellular fluids enhance a tendency to edema. On the other hand, the addition of KCl (a salt which does not accumulate in the extracellular fluid) in the same molecular concentration had a slight retarding effect (exps. 8 and 9, series 2), which may or may not be of significance.

In experiments not recorded in the graphs, addition of 5% NaCl to the low salt diet 32 at the fifty-second day of feeding regularly precipitated (within 3 days) pericardial exudates not observed in those members of the group given no additional NaCl. A few instances of such exudates were induced by 5% NaCl even when the diet contained amounts of d, l- α tocopherol (10 mg. %) usually sufficient to prevent exudative diathesis. The pericardial type of exudate appearing rapidly after the feeding of high NaCl is definitely unlike the exudative symptoms developing more slowly on diets containing a more normal salt content.

⁵ It will be noted that the experiments recorded in series I took a much longer time than all the following experiments. The reason for this is that in the first series of experiments the yeast was not extracted with ether.

Effect of histamine, etc.

Addition of traces of histamine (25 mg. per kilogram) to the exudate-producing diet with low salt content accelerated the symptom (exp. 5), yet single intravenous injections of shock-producing but sublethal doses of histamine (1 ml. of a 1.25% solution in 0.9% saline) or peptone (5.5 ml. of a 20% solution in 0.9% saline) per 300 gm. body weight failed, even though a transient edema was produced by peptone injection. Addition of histamine (100 mg. per kilogram of diet) did not accelerate the symptoms when there was no fat in the diet.

Symptoms in birds receiving the low salt diet were not accelerated by pinching wings or legs, or by injecting (subcutaneously, intramuscularly or intravenously) 1-2 ml. of the exudate, or by repeatedly injecting 1 ml. of a solution of 1% casein in alkali adjusted to pH 7.3.

Type of carbohydrate

In other experiments not recorded in the graphs the 54.6% sucrose in diet 182 was replaced by either fructose, glucose, cornstarch, alcohol-extracted cornstarch, lactose or galactose. Sucrose and fructose and perhaps glucose gave somewhat more severe symptoms than cornstarch. The effect of the latter was apparently unaffected by alcohol extraction, which removes about 0.6% of partly fat-soluble material. Neither exudates nor encephalomalacia were observed when the diet contained 54.6% lactose, a sugar which is poorly absorbed from the intestine of chicks (cf. Lenkert and Becker, '39), gives poor growth (average weight 69 gm. at 4 weeks) and causes profuse diarrhea when given in such high amounts. Galactose at the same level induces severe cramps and death within a week, associated with high blood sugar (but without any significant change in fermentable blood sugar or muscle glycogen) and almost complete absence of liver glycogen. However, a moderate amount of galactose (10% for instance) can be incorporated in diet 182 without harm and without influencing the symptoms. During the feeding hours sucrose, fructose, lactose and galactose gave reducing sugars in the urine, collected after ligation of the large intestine just superior to the cloaca and rinsing of the latter.

Type of protein

Alcohol-extracted meat meal has been used instead of alcohol-extracted casein with the same effect (Dam and Glavind, '39b). Although extraction of the casein is not absolutely necessary, it increases somewhat the incidence of exudates. Commercial desiccated

chicken eggwhite, non-extracted or extracted with ether or alcohol, and supplemented with biotin⁶ proved unsuitable for the development of exudative diathesis, the reason for which is not clear. Since addition of casein (2%) to the eggwhite diet did not increase the incidence of the symptoms, the role of a foreign protein in their production seems unlikely.

The protein-carbohydrate ratio definitely influences the tendency for the exudates to appear. With cornstarch replacing sucrose in the basal diet 182, a casein-cornstarch ratio of 15% : 54.6% (exp. 10) caused an earlier onset of exudates than a ratio of 7.5% : 62.1% (exp. 11). It is possible that retarded growth on the low protein diet, giving an average weight of 74 gm. at 4 weeks compared with 158 gm. for the diet with 15% casein, contributed to the slower development of the symptom, but a casein-starch ratio of 35% : 34.6% likewise retarded the exudates (exp. 12) even though growth was slightly improved (to 166 gm. at 4 weeks). There is apparently a certain protein-carbohydrate ratio which is optimal for the development of the symptom. As was the case in the experiments with the salt mixture, this finding may also have its parallel in clinical medicine where it is common experience that a low carbohydrate diet tends to counteract a tendency to edema.

How far the yeast or other natural source of the B-complex such as skimmed milk powder can be replaced by a mixture of the known B-vitamins without influence on the symptom remains to be investigated.

Type of fat

The fat content of the diet exerts a marked influence on the appearance of both types of symptoms. Their reported acceleration by fat (Dam, Glavind, Prange and Ottesen, '41), attributed to the highly unsaturated fatty acids present (Dam, '43), is amply confirmed by the present studies (series 5). Replacing cod liver oil in diet 182 with a concentrate of vitamins A and D⁷ caused no symptoms (exp. 15), whereas with 5% cod liver oil (exp. 16) and the fatty acid fraction of 5% cod liver oil (exp. 17) the symptoms came as usual; the non-saponifiable fraction having no such effect (exp. 18). Also ineffective was 5%

⁶ Smaco no. 200, from S.M.A. Corporation, Chagrin Falls, Ohio.

⁷ In the experiments recorded in series 4 all groups of chicks received concentrates of vitamins A and D by pipette twice weekly, the amount corresponding to 68 units of A and 5 chicken units of D dissolved in 7 mg. cod liver oil per animal per day. In all other series which included diets without cod liver oil a similar or a higher amount of A and D was given in the same manner, the solvent being either cod liver oil or oleic acid in the amount mentioned. It appeared to be of no importance which solvent was used as long as the amount was very low.

commercial oleic acid freed from traces of non-saponifiable matter (exp. 20), whereas fatty acids from linseed oil similarly purified resembled cod liver oil fatty acids in their effect (exp. 19). The observation, not recorded in the graphs, that the addition of 5% cod liver oil to the diet of chicks reared for 4 weeks on diet 182 minus this oil precipitated exudates 4 to 7 days later indicates that a certain time is required to induce the capillary damage. The symptoms produced by 5% lard were slower in their appearance but no less severe than those induced by 5% cod liver oil (exps. 13 and 14). It has further been observed that 5% commercial C₂₀ unsaturated fatty acids⁸ (iodine value 235) also accelerate exudates even though they retard body growth (to 83 gm. at 4 weeks). There seems to be little doubt, therefore, that it is the highly unsaturated fatty acids which accelerate the exudative diathesis.⁹

It is well known that cod liver oil exerts a muscular dystrophy-inducing action in rabbits which is accentuated by rancidity changes in the oil and reduced or abolished by hydrogenation (McCay, Paul and Maynard, '38; Mattill and Golumbic, '42; Mackenzie, Mackenzie and McCollum, '41), leading to the hypothesis that auto-oxidation of the fatty acids in cod liver oil destroys traces of vitamin E in the diet (and perhaps also in the body). That cod liver oil exerts a direct damage on tissues seemed unlikely, since muscular dystrophy occurs in rabbits fed an E-deficient diet exceedingly low (0.05%) in animal fats (Mackenzie, et al., '41). Since fat in the diet is prerequisite to the marked development of E-deficiency symptoms in chicks, and since previous studies (Dam, '43) indicate that the exudate-inducing action of fresh cod liver oil is unchanged by slight rancidity but abolished by thorough rancidity, the effect of fats and of rancidity upon dystrophy in rabbits appears to have no parallel in the exudative diathesis in chicks.

Additional studies reported here, carried out under slightly different conditions, further confirm these findings. Without cod liver oil no symptoms occurred (exp. 23). With fresh cod liver oil (iodine value 173) exudates developed as usual (exp. 24). With cod liver oil made thoroughly rancid in the diet by exposing the latter in thin layers for 1 week at 50°C., reducing the iodine value to 62, no symptoms occurred (exp. 25). Separate daily addition of the yeast to the diet after rancidity was developed gave the same result (exp. 26). Likewise, no symptoms appeared when the diet contained cod liver oil which had been heated in a rapid current of air at 100°C. for 1 week, reducing the iodine value

⁸ Obtained from Armour and Company, Chicago.

⁹ The basal diet 182 minus cod liver oil, which contains only traces of fat in the extracted yeast and casein (Mackenzie et al., '39), has produced exudates in only 3 out of 100 chicks. Two of these exudates were not accompanied by reddening of adipose or muscle tissue.

to 86 (exp. 27). Although in the latter experiment polymerisation of the oil may have occurred and interfered with its intestinal absorption, this series of experiments clearly demonstrates that thorough rancidity of the cod liver oil developing in the diet abolishes its accelerating effect on the symptoms.¹⁰ This effect of cod liver oil (or highly unsaturated fatty acids) on exudative diathesis must therefore be due to changes independent of destruction of traces of vitamin E in the diet through rancidity of the oil. The possibility that rancidity of the oil leads to the formation of a factor which protects against the symptoms is excluded because the addition of 5% fresh cod liver oil to the rancid diet resulted in appearance of exudates just as when no rancidity was present (exps. 28 and 24). The general trend of these experiments is in agreement with the observations that various hydroquinones which may act as antioxidants do not prevent the exudative diathesis in chicks (Bird, '43) and that the antioxygenic activity of a given compound is not a measure of its vitamin E activity (Golumbic, '41). It is also in accord with the fact that cod liver oil has been used as solvent for free tocopherol in experiments with chicks (Bird and Culton, '40) and with rats (Bacharach et al., '37; K. E. Mason, personal communication) without destroying the protective effect of the tocopherol.¹¹

Cholesterol and lipotropic substances may influence the effect of cod liver oil on the exudates (Dam and Glavind, '42). Thus the addition of 1% cholesterol to diet 32, which in itself gives a slow development of the symptoms, will cause the symptoms to appear more rapidly, whereas addition of 1.5% inositol or 2% lipocaic¹² to diet 182 resulted in marked retardation. The experiments on which the preliminary report was based are presented in series 1 (exps. 2 and 6), and series 7 (exps. 29 to 31). It was found later that cholesterol had no accelerating effect when cod liver oil was omitted from the diet. It has also been found that mannitol, sorbitol, and dulcitol do not possess the exudate-retarding property of inositol.

¹⁰ On an earlier occasion Dam and Glavind ('39) thought that rancidity might favor exudates because, in a period where the diet unintentionally had become rancid due to lack of cleanness, chicks which received a supposedly vitamin K free diet showed exudates before hemorrhages were observed. However, this must have been due to the fact that the rancidity was not complete and that simultaneous putrefaction of the casein delayed the decline of prothrombin so that the exudates appeared before the hemorrhages.

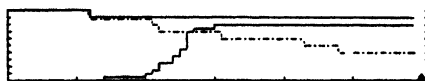
¹¹ That tocopherol acetate can be incorporated in diets with 5% cod liver oil without losing its protective effect is not surprising because the acetate is less easily oxidized than the free compound.

¹² From Eli Lilly and Co., Indianapolis. The absence of tocopherol from the lipocaic preparation was ascertained by hydrolysis of 2 gm. with 60% KOH in absence of air extraction, washing and evaporating under the same conditions and carrying out the Emmerie and Engel test as described by Dam, Glavind, Prange and Ottesen ('41).

FACTORS AFFECTING THE APPEARANCE OF ENCEPHALOMALACIA

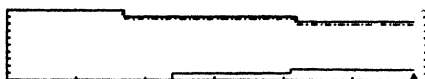
The Pappenheimer and Goettsch diet 108, hitherto used extensively for the study of encephalomalacia, contains large amounts of lard and moderate amounts of carbohydrate in the form of cornstarch. It also contains lactose from skimmed milk powder but, as previously mentioned, this carbohydrate is poorly utilized by chicks and, therefore, can be left out of consideration. With this in view it is possible to modify the exudate-producing diet 182 so that encephalomalacia appears as the chief symptom. Series 9 shows experiments of this kind.

SERIES 1.

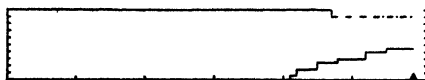


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Exp. 1. Diet 182 with unextracted yeast.



Exp. 2. Diet as in exp. 1 + 2 % lipocalc.



Exp. 3. Diet 32 (2 % salts # 3).



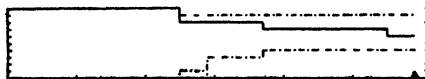
Exp. 4. Diet 32 + 2.9 % NaCl.



Exp. 5. Diet 32 + 2.5 mg% histamine dihydrochloride

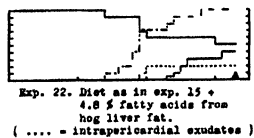
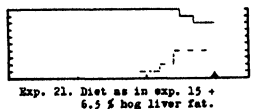
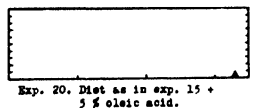
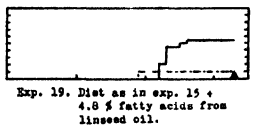
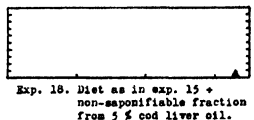
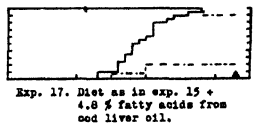
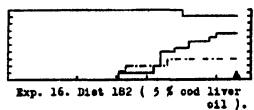
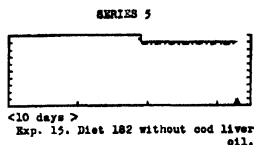
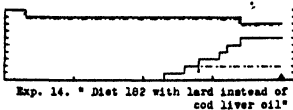
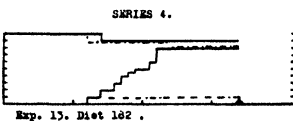
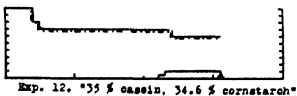
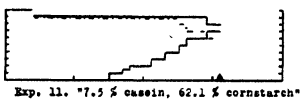
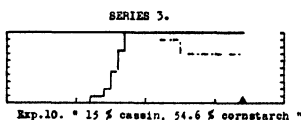
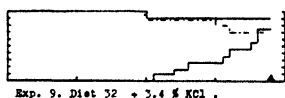
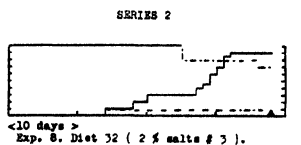


Exp. 6. Diet 32 + 1 % cholesterol.



Exp. 7. Diet 32 with dried hog liver instead of casein.

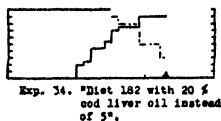
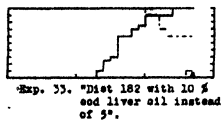
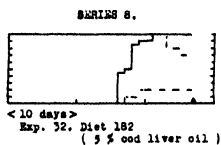
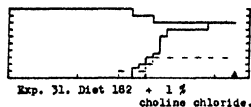
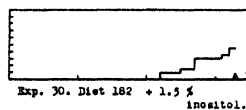
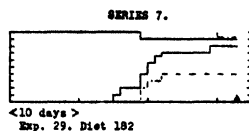
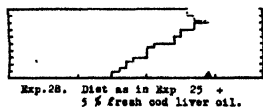
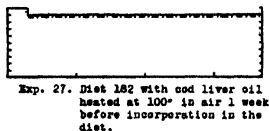
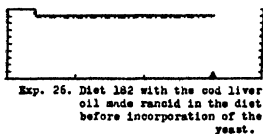
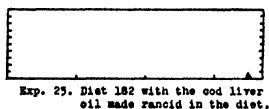
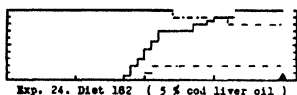
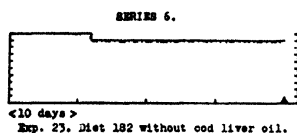
Cod liver oil was omitted in order to make the experiments simpler and the variations of the diet consisted in changing the lard and carbohydrate as indicated in the legend, the other constituents being unaltered.



Fat, protein and carbohydrate

With 30% lard replacing the same amount of sugar, whereby the sugar was reduced to 24.6%, both exudates and encephalomalacia developed about equally; encephalomalacia appeared slightly in advance of the exudates (exp. 35). With 30% lard and 24.6% cornstarch (diet 190, table 1), encephalomalacia was by far the dominating symptom, exudates coming decidedly later (exp. 36). Encephalomalacia was still more favored when all the carbohydrate of the diet containing 30% lard was replaced by casein, the total casein content being 39.6% (exp. 37).

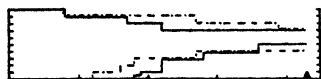
Without lard (exps. 38 and 39) no encephalomalacia appeared but one instance of exudate, rarely seen on "fat-free" diets, did occur (exp. 39). It is interesting to compare the latter with experiments 40 and 41,



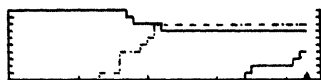
series 10, in which the diets contained 5% cod liver oil. When in diet 182 the sugar was removed and the casein increased to 35%, with the simultaneous introduction of 35% lard, the symptom shifted from exudate to encephalomalacia, the body weight increasing to 265 gm. in 4 weeks.

A similar shift occurred when cod liver oil or its fatty acids in diet 182 (exps. 16 and 17) were replaced by hog liver fat or its fatty acids (exps. 21 and 22), and when dried hog liver replaced casein as the source of protein in diet 32 (exp. 7). Hog liver fatty acids produced intra-pericardial exudates in two chicks (exp. 22).

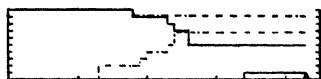
SERIES 9.



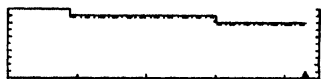
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Exp. 35. " casein 15, sucrose 24.6, lard 30 "



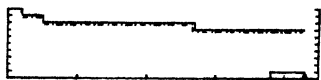
Exp. 36. Diet 190. " casein 15, alcohol-extracted cornstarch 24.6, lard 30 "



Exp. 37. " casein 39.6, lard 30, sucrose 0 "



Exp. 38. " casein 39.6, sucrose 30, lard 0 "

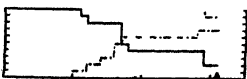


Exp. 39. Diet 182.
" casein 15, sucrose 54.6, lard 0 "

SERIES 10.



<10 days >
Exp. 40. Diet 182
" casein 15, sucrose 54.6, lard 0 "

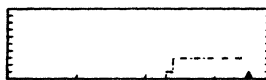


Exp. 41.
" casein 35, lard 35, sucrose 0 "

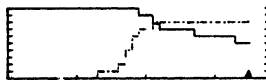
SERIES 11.



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Exp. 42. Diet 190.



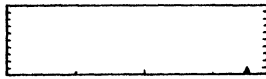
Exp. 43. Diet 190 + 1.5 % inositol.



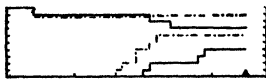
Exp. 44. Diet 190 + 2 % linolenic.



Exp. 45. Diet 190 + 1 % cholesterol.



Exp. 46. Diet 190 + 10 mg% d,l-alpha-tocopherol acetate.



Exp. 47.
" Diet 190 with 2 % salts # 3 instead of 7.2 % of the prescribed salt mixture "

Not only cod liver oil (exp. 16) but also linseed fatty acids (exp. 19) and lard (exp. 14) caused some encephalomalacia when constituting about 5% of the diet, while commercial oleic acid (exp. 20) or thoroughly rancid cod liver oil (exps. 25-27, series 6) did not. Furthermore, increasing the cod liver oil in diet 182 from 5% to 20% (exps. 32-34, series 8) did not increase the incidence of encephalomalacia but may have reduced it.

The data thus far collected show that certain fats will favor exudates whereas others will favor encephalomalacia. Whether this is due to the fatty acids themselves or to trace substances which go into the fatty acid fraction remains to be definitely settled. Encephalomalacia has never been observed, and exudates rarely seen, when fat was not added to the diet. Furthermore, low carbohydrate or the use of a moderate amount of starch as the source of carbohydrate instead of sucrose will depress the tendency for the exudates to appear and favor encephalomalacia, provided sufficient fat such as 30% lard is present.

Cholesterol and lipotropic substances

The experiments included in series 11 demonstrate that both encephalomalacia and exudates were counteracted by 1.5% inositol (exp. 43), only exudates by 2% lipocaic (exp. 44), and only encephalomalacia by 1% cholesterol (exp. 45); whereas 10 mg. % of α -tocopherol prevented both symptoms (exp. 46). Changing the salt mixture to 2% of salts no. 3 (exp. 47) had no significant influence except that growth was retarded (average weight of 126 gm. at 4 weeks, compared to 175 gm. with diet 190 and 200 gm. with diet 190 plus inositol). It is of interest to note that with diet 182 containing 5% cod liver oil, 2% lipocaic likewise suppressed exudates (exp. 2) whereas 1% cholesterol added to diet 32 exerted an accelerating effect upon exudates (exp. 6) which did not occur with diet 190 (cf. exps. 45 and 42). From the foregoing it is apparent that substances influencing fat metabolism will also influence the nature and incidence of the symptoms. Obviously the problems raised by these observations require further investigation with varying amounts of the substances tested.

The failure of the Pappenheimer and Goettsch diet 108 to induce exudates has been explained on the assumption that certain ingredients of the diet provide traces of vitamin E sufficient to prevent this symptom but not adequate to meet a supposedly higher requirement for the prevention of encephalomalacia (Dam and Glavind, '39b). The results of the present studies indicate that the low carbohydrate content of this diet in itself tends to delay or minimize the tendency to exudates.

DISCUSSION

One might ask whether encephalomalacia is not simply due to exudation in brain tissue. Several of the above mentioned facts obviously speak for this view. However, the question is complicated by the fact that there is no complete parallelism between the factors which favor or counteract the two symptoms.

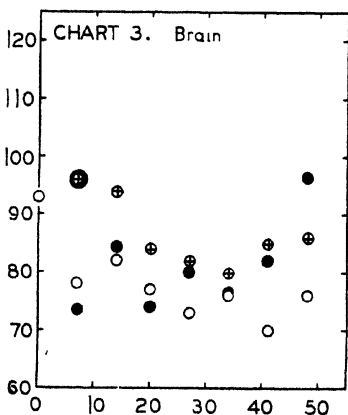
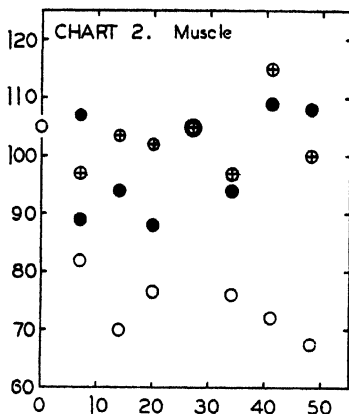
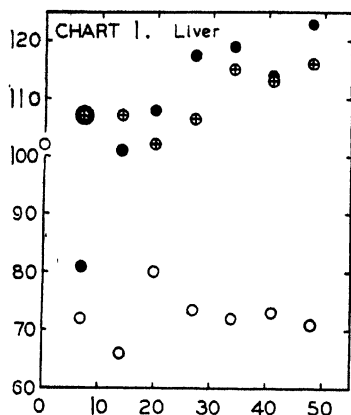
If fats merely destroy vitamin E in the body, it is difficult to explain why some fats favor exudates and others encephalomalacia, and why dietary fat is so essential for the development of symptoms in chicks but not so in rabbits. Certain fatty acids undoubtedly exert a damaging effect on the affected tissues, directly or indirectly but the nature of this mechanism remains to be investigated. Vitamin E deficiency seems not to incur a general inability to metabolize fat as manifested by ketonuria, lipemia or fatty liver. There is some evidence (Dam and Kelman, '42) that a deficiency of vitamin E decreases the ratio of phospholipids to other lipids in the blood plasma of chicks but the difference (20-40%) is not great enough to be of diagnostic value because of the considerable individual variation. The present studies have revealed no significant alteration in tissue lipids of deficient chicks, except for an increase in muscle cholesterol (170 mg. % with diet 182 against 104 mg. % when the diet contained 10 mg. % d, l- α -tocopherol acetate); a finding in harmony with observations on rats (Heinrich and Mattill, '43). Tests for rancidity in the fat tissue were negative.

Sinclair ('32) has shown that in rats the phospholipids take up highly unsaturated fatty acids from the diet. In order to find out whether vitamin E has any gross influence on this process I determined the iodine value¹³ of the phospholipids of liver, muscle and brain from chicks reared on diet 182, diet 182 + 10 mg. % d, l- α -tocopherol acetate, and diet 182 without cod liver oil (vitamins A and D concentrate being given to all groups). Charts 1, 2 and 3 show that while there was a considerable difference in iodine value of liver and muscle phospholipids according to whether cod liver oil was given or not, the presence or absence of vitamin E did not cause any significant difference. It has further been shown that the iodine value of the subcutaneous depot fat is not significantly influenced by the presence or absence of vitamin E.

¹³ The organs were ground with sodium sulfate immediately after killing by decapitation, the lipids extracted with chloroform at room temperature for 24 hours, evaporated in vacuo at body temperature and extracted six times with acetone. After dissolving the residue in chloroform the dry weight was determined in one aliquot and the iodine value in another, the method being that of Rosenmund and Kuhnemann as described by Dam ('30).

Regarding the modifying influence of inositol, lipocaeic, cholesterol, carbohydrates, and protein-carbohydrate ratios, it may be concluded that this influence is too complicated to be explained by general pro- or anti-oxidant effects.

Some observations relative to the response of rats to diets which produce exudates or encephalomalacia in chicks warrant brief mention here. Rats showed no such symptoms when fed a vitamin E deficient



Abscissa: Age in days.

Ordinate: Jodine value of acetone-insoluble lipids

- ⊕ Diet 182
- Diet 182 + 10mg% d,l- α -tocopherol acetate
- Diet 182 without cod liver oil

diet from the time of weaning, even if the amount of cod liver oil in the diet is high. However, a peculiar buff discoloration of the subcutaneous and intraperitoneal fat tissue occurred without any signs of hemorrhage in (a) rats reared for 183 days on diet 182, to which was added 1% cholesterol, 1% NaCl and 60 mg. % of histamine dihydrochloride, and in (b) rats reared for 111 days on diet 182, modified by increasing the

cod liver oil from 5% to 20%. This change of the fat did not occur either in controls fed the same diets + 10 mg. % d, l- α -tocopherol acetate or in rats fed the unsupplemented diet 182 containing only 5% of cod liver oil. Should it be possible to demonstrate that this phenomenon is related to the buff coloration which develops in fat tissue in chicks receiving diet 182, after the reddishness of the fat tissue has disappeared, there would exist a bond of similarity in the response of these two species to vitamin E deprivation. In this connection it may be noted that a hemorrhagic tendency has been observed in the fetus of rats receiving a vitamin E low diet (Mason, '42, '43), which may have some relation to the exudative diathesis in chicks.

Rabbits will develop muscular dystrophy when placed on various vitamin E deficient diets, including the Pappenheimer and Goettsch diet 108, but nothing resembling exudates or encephalomalacia has been observed in them. Pappenheimer and Goettsch ('34) made the surprising observation that diet 108 also produces only muscular dystrophy in ducklings. Ten newly hatched ducklings reared for 33 days in the author's laboratory on the usual exudate-producing diet 182 did not show any definite signs of exudate or encephalomalacia. The reason for this different behavior of different species even when they are so closely related as ducks and chicks has not been explained, but it would not seem unlikely that differences in the fat-metabolizing enzyme system may play a rôle.

Symptoms corresponding to exudative diathesis and encephalomalacia have not been recognized in humans. The possibility that vitamin E might influence the capillary phenomena in certain forms of allergy was examined by Glaser and Dam ('44) with negative results.

SUMMARY

The symptoms of vitamin E deficiency in chicks — exudative diathesis and encephalomalacia — can be accelerated or suppressed by dietary changes which are unrelated to the vitamin E content of the diet.

Purified diets containing no added fat rarely produce exudates and never induce encephalomalacia. Both symptoms are accelerated by highly unsaturated fatty acids.

The fatty acids from cod liver oil, lard and linseed oil and commercial unsaturated C₂₀ acids at a level of about 5% in the diet produce exudates as the main symptom, whereas fatty acids from hog liver favor the appearance of encephalomalacia. Oleic acid and thoroughly rancid cod liver oil are ineffective in producing either symptom.

A certain protein-carbohydrate ratio is optimal for the appearance of exudates, and the tendency to exudates is further enhanced by those inorganic salts which tend to accumulate in the extracellular fluid.

High lard (30%) favors encephalomalacia more than exudates when the carbohydrate-protein ratio is low. Inositol (1.5%) counteracts both symptoms. Lipocaic (2%) counteracts only exudative diathesis. Cholesterol (1%) hastens exudates when the diet contains 5% cod liver oil and a low salt content, and counteracts encephalomalacia when the diet contains 30% lard. The iodine value of body phosphatides is influenced by dietary fat independently of the presence of vitamin E.

It is believed that the effect of the different fatty acids consists in a damage of tissue rather than in a general destruction of vitamin E, and that inositol, lipocaic and cholesterol do not act simply through a general pro- or anti-oxidant effect.

The response of different species of animals to diets which produce exudates or encephalomalacia in chicks is discussed.

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NUTRITIONAL REQUIREMENTS OF THE SYRIAN HAMSTER

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Routh and Houchin ('42), in a preliminary report, stated that hamsters require thiamine, riboflavin, pyridoxine, pantothenic acid, and nicotinic acid. More recently Cooperman, Waisman, and Elvehjem ('43) have described a study of the requirement of these animals for the water soluble vitamins. The hamsters grew at a normal rate when the diet was supplemented with nine members of the vitamin B complex, including thiamine, riboflavin, calcium pantothenate, pyridoxine, nicotinic acid, sodium para-aminobenzoate, inositol, choline, and biotin. A few females were carried through reproduction with fair success. The authors concluded that the hamster does not require nicotinic acid. They were uncertain whether both inositol and para-aminobenzoic acid are required, but they were convinced that at least one of these is required. Our observations on the nutritional requirements of these animals are described in the following pages.

EXPERIMENTAL

Since many investigators are not familiar with the habits of hamsters² a brief description of the routine management of our colony is included. Ordinarily the animals are quartered in square cages of $\frac{1}{2}$ inch mesh hardware cloth, with floors of this same material. The food is supplied ad libitum in glass jars, and water is provided from inverted bottles with glass drinking tubes. Hamsters are usually gentle and easily handled, though on rare occasions a female has become vicious. As the animals approach sexual maturity the males and females are segregated, with three or four together to a cage. The females, especially when pregnant, are much more pugnacious than the males and if caged together the males may be badly scarred. For convenience the breeding animals are usually confined in individual cages.

¹ Contributions from Missouri Agricultural Experiment Station, journal series no. 911.

² Our foundation stock was purchased from Henry Bergman, Springfield, Mo.

When the females are 8 or 9 weeks of age they are mated to a male not closely related and at least 12 weeks of age. The females are placed in the cage with the male and are removed after 5 minutes if not receptive and after 10 or 15 minutes if receptive. On the thirteenth day of gestation the floors of the cages are removed and a small quantity of shredded paper is provided for a nest. The females are disturbed as little as possible on the sixteenth day and for the first 2 days following birth of the litter. If a female is disturbed shortly after parturition she almost invariably becomes excited and kills and eats some of her litter. The young are not handled until 1 week of age, and are weaned when 21 days of age. The females are given a 2-week rest period between the weaning of a litter and remating, and are retained until approximately 1 year of age when their usefulness declines. Female hamsters attain a mature weight of 105–135 gm. while the males are somewhat smaller, weighing between 95 and 120 gm.

The composition of the stock ration, no. 1, is shown in table 1. Each female, during lactation, receives in addition 10 ml. of whole milk

TABLE 1
Composition of diets.

<i>Ration</i>	<i>%</i>		<i>%</i>
1 Ground wheat	28	Ground corn	20
Wheat germ	7	Dried yeast	10
Alfalfa meal	5	Calcium carbonate	1
Dried skim milk	15	Sodium chloride	1
Linseed oil meal	12	Vitamin A-D mixture ¹	1
<i>Basal mixture</i>		<i>Vitamins mg per 100 gm of basal mixture</i>	
2 Casein (acid washed and alcohol extracted)	20	Thiamine hydrochloride	0.8
Cerelose	65	Riboflavin	1.6
Lard	7	Pyridoxine hydrochloride	1.2
Cellulose	3	Calcium pantothenate	1.0
Salt mixture ²	4	Nicotinic acid	5.0
Vitamin A-D mixture ¹	1	p-Aminobenzoic acid	100.0
		Inositol	250.0
		Choline chloride	400.0
		2-Methyl-1, 4-naphthoquinone	3.0
		Alpha tocopherol	2.5

¹ Composed of a mixture of 2 parts Mead-Johnson oleum percomorphum and 98 parts of lard. One gram of the mixture supplies 1200 I.U. of vitamin A and 170 I.U. of vitamin D.

² A modification of mixture 351 of Hubbell, Mendel, and Wakeman ('37), in order to increase the phosphorus content:

CaCO ₃	125.2	KH ₂ PO ₄	212.0
Ca ₁ (PO ₄) ₂	376.3	FePO ₄ · 4H ₂ O	20.5
MgCO ₃	25.0	MnSO ₄ · 4H ₂ O	25.5
MgSO ₄ · 7H ₂ O	32.8	CuSO ₄ · 5H ₂ O	1.4
NaCl	69.0	Al ₂ (SO ₄) ₃ · K ₂ SO ₄ · 24H ₂ O	0.17
KCl	112.0	KI	0.08

daily. Several females have borne and reared five litters while consuming the stock ration, and in no cases were nutritional deficiencies observed. The heaviest mortality takes place during the first 5 days of life and is especially heavy during the first 2 days. The fact that the young are not counted until the second day after birth accounts for the relatively small litter size.

GROWTH ON SIMPLIFIED RATIONS

Adequacy of synthetic vitamins^a

The first step was to determine whether a simplified ration containing only the known vitamins is nutritionally adequate for growth. The first ration studied is very similar to the one that was shown by Richardson, Hogan, Long, and Itschner ('41) to be adequate for growth of the albino rat. The composition of diet no. 2 is given in table 1. The simplest experimental diet contained the six vitamins, A, D, thiamine, riboflavin, pyridoxine, and calcium pantothenate. For convenience the other vitamins included in the various diets are given in table 2.

The amounts of the vitamins in the various diets, if included at all, were the same as in ration 2. No effort was made to determine the

TABLE 2

Vitamins included in rations.

RATION NUMBER	NICOTINIC ACID	CHOLINE	INOSITOL	P-AMINO- BENZOIC ACID	BIOTIN	VITA- MIN B	VITA- MIN K
Rations used for studies on growth							
2	+	+	+	+	..	+	+
3	—	+	+	+	..	+	+
4	—	—	—	—	..	+	+
5	+	+	+	+	..	—	+
6	—	—	—	—	..	—	+
7	+	+	+	+	..	+	—
8	—	—	—	—	..	+	—
9	—	—	—	—	..	—	—
Rations used for studies on reproduction							
2	+	+	+	+	—
10	+	+	+	+	+
11	+	+	+	—	+
12	+	+	+	—	—
13	+	—	+	—	—
14	—	+	+	—	—
15	—	—	+	—	—
16	+	+	—	+	+

^aSupplied by Merck and Co., Rahway, New Jersey.

minimum requirement for any of them and in all probability the quantities supplied were larger than necessary. The average weekly weights of the animals on the various rations are shown in table 3. The rate of growth and the mature weights of animals that consumed ration 2 were equal to those of the stock animals. Hamsters grow at a normal rate on diets that contain no vitamins except those now recognized.

TABLE 3
Growth of hamsters on simplified diets.

Ration no.	COLONY DIET		SIMPLIFIED DIETS						
	1		2	2a	3	4	7	8	
No. of animals	12F	11M	5F	4M	5M	9M	10M	3F	8M
Age weeks	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
3	27	29	28	28	24	27	26	27	26
4	44	42	41	37	37	39	36	37	36
5	50	55	54	49	53	48	44	49	46
6	60	63	66	60	63	62	50	58	53
7	69	70	76	70	74	71	47	61	62
8	77	76	84	78	82	77	47	75	67
9	86	82	89	85	87	86	60	81	75
10	93	85	96	91	89	91	72	89	81
11	101	86	103	93	90	93	77	94	83
12	105	90	100	96	94	93	80	97	81
13	111	93	94	100	96	95	82	100	91
14	108	96	100	101	97	96	87	101	93
15	112	98	107	102	101	99	92	105	93
16	115	96	106	100	104	99	95	101	91
17	113	103	110	101	104	101	95	105	94
18	115	100	111	100	104	101	95	103	94
19	120	102	117	100	103	102	96	104	94
20	117	105	120	100	102	101	100	...	93
21	123	106	121	101	102	104	101	...	93
22	113	109	119	100	104	106	102	...	95
23	110	106	119	103	106	107	105	...	97
24	111	108	122	103	109	109	107	...	97

Nicotinic acid

Ration 2a contains Labco⁴ vitamin-free casein but in all other respects is identical with ration 2. Ration 3, except for the omission of nicotinic acid, is identical with ration 2a. The animals which consumed these two rations grew at practically the same rate and no abnormalities were observed. They were indistinguishable from stock animals of the same age and it seems certain that under our experimental conditions hamsters do not require dietary nicotinic acid for growth.

⁴ Purchased from the Casein Co. of America, New York, New York.

Choline, inositol and p-aminobenzoic acid

An attempt was then made to simplify the diet still further and ration 4 was prepared which contains the four fat-soluble vitamins A, D, alpha-tocopherol, and 2-methyl-1, 4-naphthoquinone, and the four water-soluble vitamins, thiamine, riboflavin, pyridoxine, and pantothenic acid. The constituents of the diet may contain other vitamins as contaminants, but biotin is the only one for which a search was made. It was estimated⁵ that the casein contained 0.0150 µg. of biotin per gram and the cerelese contained 0.0042. These two components would supply 5.73 µg. of biotin per kilogram of ration. Since the chick requires several times this amount, one would expect ration 4 to be inadequate for growing hamsters if they require dietary biotin. However, the animals on this diet grew rapidly and reached a normal mature weight. It was concluded that it is unnecessary to include either choline, inositol, or para-aminobenzoic acid in the diet of hamsters during the stage of growth.

Alpha-tocopherol

Two vitamin E-free diets, nos. 5 and 6, were used to determine whether this vitamin is essential for the hamster, but the animals responded in the same manner to both rations. The growth rate was normal at first, then gradually declined. The animals rarely exceeded 85 gm. in weight and invariably lost weight before death. Of the twenty-eight animals on these rations all collapsed. Six were treated and recovered and the other twenty-two died. The animals exhibited no abnormal symptoms, other than slow gain, or loss in weight, until less than 2 hours before death. The first abnormal symptoms were unusual activity, lack of coordination, and variable activities such as jumping in the air, rolling over, biting themselves, running about the cage, copious flow of saliva, and violent reaction to external stimuli such as touch or noise. These manifestations increased in intensity and approximately 1 hour before death the animals collapsed, lost consciousness, and lay in a stupor. Seven of the animals in this final stage were each treated with 5 mg. of alpha-tocopherol. One died within 10 minutes after treatment and the other six made spectacular recoveries. They regained consciousness within 45 minutes, and within 1 hour they were able to move about the cage, and after 12 hours they were eating. At this point alpha-tocopherol was included in their diets and thereafter they gained rapidly and those that were retained long enough reached

⁵ The assays were made by Mrs. Barbara Long in Dr. V. du Vigneaud's laboratory, Cornell Medical College, New York, New York.

normal mature weights. Autopsy of the animals that died did not disclose any gross abnormalities but no histological examinations were made. The depletion periods before collapse varied from 4 to 18 weeks, but eventually all animals were affected. There is every reason to suppose the hamster could be used conveniently for the biological assay of alpha-tocopherol.

Vitamin K

Diets 7 and 8 did not contain 2-methyl-1, 4-naphthoquinone, and they were used to study the requirement of the hamster for vitamin K. The weanling hamsters grew normally for 2 or 3 weeks on both rations and then the growth rate gradually declined. Somewhere between the third and seventh week all of them either gained slowly, ceased to gain, or lost up to 20% of their body weights. During this period of arrested growth they became thin and presented an unthrifty appearance. Three animals at this stage were sacrificed for examination. All three had a considerable number of small hemorrhagic areas in the muscle and subcutaneous tissues and in the lining of the abdominal cavity. Free blood was also found in the sinuses. These hemorrhages were not fatal but they did retard the rate of growth or even caused a temporary loss in body weight. Following the period of arrested growth the animals grew rapidly and attained normal mature weights at approximately the same age as did similar animals receiving rations that contained vitamin K. The resumption of growth is probably explained by the establishment of intestinal flora of a type that can synthesize vitamin K to prevent hemorrhages.

Vitamins E and K

Several weanling hamsters were placed on ration 9 which contained neither vitamin E nor vitamin K. The growth rate on ration 9 was retarded to about the same extent as on the E deficient rations previously described. Of a total of seventeen hamsters, male and female, which received ration 9, fourteen died after depletion periods that varied from 6 to 16 weeks. The symptoms preceding death were the same as on rations 5 and 6, but autopsy revealed the hemorrhages that are characteristic of a vitamin K deficiency. Three of the animals were found in the unconscious stage that precedes death and 5 mg of alpha-tocopherol were administered to each. All three made rapid recoveries.

Vitamins required for growth

Other data, which will not be described, agree with Routh and Houchin ('42) in showing that the hamster requires thiamine, ribo-

flavin, pyridoxine, and pantothenic acid. The requirement of this animal for vitamins A and D has not been investigated, but it is assumed for the present that both are required. Vitamin E is essential and vitamin K must also be supplied if a normal rate of growth is to be maintained.

REPRODUCTION

Colony ration

The litter records are summarized in table 4. It will be observed that each of 6 females on ration 1 reared four litters, and that three of them

TABLE 4
Litter records.

	RATION AND LITTER NO.	NO OF LITTERS	NO OF YOUNG PER LITTER	NO. OF LITTERS WEANED	NO. OF YOUNG WEANED PER LITTER	WEANING PER- CENTAGE	AVERAGE WEAN- ING WEIGHT	DAYS PER LITTER ¹	NO. OF FEMALES AT CLOSE OF EXPERIMENTAL PERIOD	DAYS FROM LAST LITTER TO CLOSE OF EXPERIMENTAL PERIOD
1.	I	10	4.4	8	2.9	52	25.0	78
	II	10	6.2	8	4.0	52	29.0	56
	III	9	6.1	7	4.7	60	26.7	55
	IV	6	4.7	5	3.4	61	28.0	57
	V	3	3.3	3	3.0	90	24.6	81	7	91
2.	I	3	4.0	3	2.0	50	23.5	41	3	57
10.	I	3	5.7	3	3.3	59	21.0	64	.	..
	II	1	6.0	1	5.0	83	31.2	54
	III	1	4.0	1	2.0	50	27.5	50	3	66
11.	I	9	3.4	5	2.8	45	21.8	32
	II	2	2.5	1	1.0	20	24.0	78
	III	1	2.0	1	1.0	50	21.0	64	8	79
12.	I	12	3.7	10	3.1	70	21.6	53
	II	5	4.2	5	3.8	90	25.3	88
	III	2	2.5	2	1.5	60	23.7	90	12	115
13.	I	9	3.8	3	1.0	9	23.3	47
	II	2	3.0	1	1.0	17	24.0	50	.	..
	III	1	8.0	0	0.0	0	..	51	7	114
14.	I	6	3.0	3	1.0	10	24.5	41
	II	1	2.0	1	2.0	100	22.0	20	4	94
15.	I	6	3.3	2	1.3	22	23.3	51
	II	3	2.3	1	1.0	14	10.0	46	6	109

¹ This column signifies the number of days that intervened between litters. Under our procedure, the minimum, except for first litters, is about 51 if each litter is weaned.

reared a fifth. The percentage of young weaned varied from 52 to 90%, and the average weaning weights, at 21 days, varied from 24.6 to 29.0 gm. This record is fairly satisfactory but in all probability it can be improved. A certain amount of time was required to become familiar with the habits of the animals and it is believed that the mortality rate has been reduced by providing a more suitable environment. Furthermore it is not certain that the colony ration is adequate for optimum nutrition.

Synthetic diets

None of the experimental diets contained less than the eight vitamins that were included in diet 4. For convenience the other vitamins included in the various diets are given in table 2.

The three females on ration 2 bore three litters, and weaned 50% of their young, but the weaning weights were lower than on the colony diet. None of the females bore a second litter within a reasonable time and they were changed to another diet. However, it was certain that hamsters could attain some degree of success in rearing litters on synthetic diets and the study was continued, with diets containing various combinations of the available vitamins.

Biotin and p-aminobenzoic acid

Three females received ration 10, which except for the addition of biotin is the same as ration 2. The amount of the vitamin^a supplied was 3 µg. daily per animal. Two of the females weaned one litter each. The third female reared three litters, and both the percentage of young weaned and the weaning weights were approximately the same as in the breeding colony on the stock diet, no. 1. Apparently the animals became depleted of another vitamin, however, for additional litters could not be secured. The response of the one animal that bore three litters gives some indication that the hamster requires both biotin and p-aminobenzoic acid for reproduction, but it may be impossible to arrive at a final decision until all other essential vitamins become available.

Ration 10 was modified by the omission of p-aminobenzoic acid to make ration 11, and by the omission of both biotin and p-aminobenzoic acid to make ration 12. The females on these two rations were about as successful in rearing litters as were those on ration 10 except the weaning weights were lower.

^aS.M.A. Corporation Conc. no. 200.

Choline and nicotinic acid

The rations used to study the requirement of the hamster for these two vitamins during the reproductive stage were modifications of ration 12. Ration 13 contains nicotinic acid but no choline. Ration 14 contains choline but no nicotinic acid. Ration 15 contains neither. The females on all three rations bore litters that were apparently normal, but the number of young per litter was unusually low. Apparently lactation was subnormal since most of the young died within 1 week, indicating that both nicotinic acid and choline are required by the hamster for successful lactation.

Inositol

The inositol-free diet used most extensively was no. 16 which, except for the deficiency in inositol, is identical with ration 10. The females on the inositol-free rations have borne twenty-five litters, but twenty of these were still-births and the embryos were bloody, shapeless masses when expelled, usually at full term. Several females died during or shortly after parturition, with one or more decomposed embryos in the uterus. There were fifteen living young in the other five litters. One of the young was weaned and another died when 20 days old. The others died, or were killed by their mothers, before they were 1 week old. We assume that the mothers which bore living young had not been sufficiently depleted of their inositol reserves.

Vitamins required for reproduction

Nearly all the females bore one litter, a few bore a second, and occasionally one would bear a third. It seems quite certain that all of the diets were inadequate for reproduction, and that the hamster requires at least one vitamin that has not been recognized as yet. When all the experimental diets are inadequate, the significance of differences between diets that contain different numbers of vitamins may be uncertain. With this reservation in mind, our data indicate that during the reproductive phase female hamsters should receive at least seven members of the vitamin B-complex. In addition to the four that are required for growth the diet should include inositol, nicotinic acid and choline. The evidence concerning biotin and p-aminobenzoic acid was insufficient to justify listing them as essential vitamins. There has been no opportunity as yet to determine whether the hamster requires vitamin B₆. (Hogan and Parrott, '40; O'Dell and Hogan, '43; Pfiffner et al., '43).

DISCUSSION

The nutritional requirements of the hamster have not been studied extensively, but the discrepancies that have appeared deserve some comment. We agree with Cooperman and collaborators ('43) that the hamster does not require nicotinic acid for growth. According to Cooperman et al. this animal requires biotin for growth but we were unable to repeat this observation. In our experience the growth rate of hamsters is improved by including vitamin K in the diet, but Cooperman et al. reported that their animals grew at a normal rate on rations that did not contain this vitamin. However, our animals consistently grew more rapidly than did those described by Cooperman and co-workers. Their male hamsters gained 1.0 gm. and the females gained 1.4 gm. daily in a 28-day period. Our males made an average gain of 1.5 gm. and the females 1.7 gm. daily, from the twenty-first to the forty-ninth day of age. The Wisconsin group also reported that the hamster requires either p-aminobenzoic acid or inositol, or possibly both, during growth. We did not find that either of these substances increased the rate of growth, though in the absence of inositol our females seldom bore living young. Thus far, biotin and p-aminobenzoic acid have not markedly improved any of the hamster diets we have tried, but a final decision is reserved until the trials can be repeated under more favorable conditions. An explanation of the discrepancies between our observations and those of Cooperman and associates, may be found in the interrelations between p-aminobenzoic acid, inositol, biotin, and vitamin K, which determine the rate at which vitamins are synthesized in the intestinal tract. Schmidt and Büsing ('42) report that when chicks receive a diet deficient in vitamin K there is a reduction in the number of intestinal bacteria. Black, Overman, Elvehjem, and Link ('42) have shown that if rats consume a synthetic diet to which sulfaguanidine has been added, they grow slowly and develop hypoprothrombinemia, presumably due to the depressed synthesis of vitamin K by intestinal bacteria. In a similar manner, a deficiency of biotin (Daft, Ashburn and Sebrell, '42; Martin, '42; Neumann, Krider and Day, '43; Nielsen and Elvehjem, '42) and of folic acid (Nielsen and Elvehjem, '42; Martin, '42) may be induced by the administration of sulfa drugs. One might speculate that when vitamin K is included in a diet such as no. 4, the synthesis of biotin, inositol, vitamin B₁₂, or other vitamins, is accelerated and it is then unnecessary to include them in the diet. Unfortunately, this investigation was interrupted before it was completed, and there has been no opportunity to reexamine the dietary role of

biotin, inositol, p-aminobenzoic acid, or of any of the more recently discovered vitamins. It is hoped that the study can be resumed at a later date.

SUMMARY

1. Hamsters grow at a normal rate and attain normal mature weights on simplified rations. The list of vitamins in the simplest satisfactory diet includes A, D, E, K, thiamine, riboflavin, pyridoxine, and pantothenic acid.

2. If vitamin E is omitted from the diet the animals collapse and die in 4 to 18 weeks. They may be rescued shortly after collapse by administration of vitamin E.

3. If vitamin K is omitted from the diet the rate of growth is irregular but the animals reach maturity in nearly normal time. During the period of arrested growth the animals develop small hemorrhagic areas.

4. If vitamins E and K are both omitted from the diet the animals apparently die from the vitamin E deficiency but they are also severely hemorrhagic.

5. A high percentage of females bore at least one litter on simplified diets that contained nicotinic acid, choline, and inositol, in addition to the vitamins required during growth. The data are insufficient to decide whether biotin or p-aminobenzoic acid are essential for the hamster.

6. Few females bore a second litter and it was concluded that the hamster requires at least one unrecognized vitamin for reproduction.

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THE EFFECT OF GERMINATION, THE STAGE OF MATURITY, AND THE VARIETY UPON THE NUTRITIVE VALUE OF SOYBEAN PROTEIN¹

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The biochemical literature contains numerous reports on the effect of heat on the nutritive value of proteins. Most of the investigators found heat to be injurious; however, in the case of legume protein, heat was found to be beneficial. The reader is referred to papers by Morgan and Kern ('34), Greaves and Morgan ('34), and Fairbanks and Mitchell ('35) for references to the effect of heat on animal protein. Results with legume protein are given in detail in papers by Hayward, Steenbock and Bohstedt ('36), and Johnson, Parsons and Steenbock ('39).

Much of the work on the effect of heat has been limited to soybeans of the Illini variety. This made it desirable to determine if this effect would be produced in other varieties as well. Since the improvement in nutritive value seemingly involved the sulfur-containing amino acids, five varieties of soybeans reported to vary widely in cystine content (Hamilton and Nakamura ('40)) were selected for the experiments. These included the Illini, Dunfield, Mansoy, Virginia Brown and Mandarin varieties.

EXPERIMENTAL

All the beans were fed in a ration of the following composition: soybeans to 10% protein ($N \times 5.7$); salts (Wesson, '32), 4%; cotton-seed oil,³ 5%; glucose⁴ to 100 parts; haliver oil, 2 drops weekly; riboflavin, 20 μ g. daily; and a commercially available rice-bran concentrate,⁵

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²Now at Wayne University, Detroit, Michigan.

³Wesson oil.

⁴Cerelose.

⁵Vitab, from the Oil Products Company, Harrison, New Jersey.

400 mg. daily. The beans were fed raw and after autoclaving for 15 minutes at 17 pounds pressure to young male rats weighing between 55 and 65 gm. The efficiency of the protein was judged by the gain in weight per gram of protein ingested during an experimental period of 8 weeks. In addition, after the animals had been on the diets for 14 days, fecal samples were collected daily over a 6-day period to determine the percentage of ingested nitrogen which was absorbed.

Tao and Kamatsu ('32) have reported that the protein of the soybean is appreciably altered by germination, the globulins undergoing a partial hydrolysis with the formation of more simple forms such as proteoses and peptones. As there was a possibility that this might affect the nutritive value of the proteins, soybeans of the Illini variety were germinated and fed before and after autoclaving. For germination they were spread in a thin layer between moist layers of burlap contained in shallow porcelain pans. Sufficient distilled water was then added six to eight times daily to keep them moist, without actually immersing them in water. Germination was continued at 26°C. for approximately 60 hours. At the end of this time approximately all of the beans had sprouts varying in length from 2 to 12 mm. The beans were light yellow in color and about four times their original size. They were ground with a meat grinder, spread out thinly in trays and dried before a fan at room temperature. They were then reground to a fine meal in a burr mill with due care to prevent heating.

Parsons ('43) has suggested that the degree of maturity of the soybean might be a factor in determining the nutritive value of its protein. However, the data obtained were limited and disregarded possible varietal differences. Experiments were therefore carried out, using only beans of the Illini variety. These beans were picked when immature and shelled without the use of heat. They were ground and then dried before a fan at room temperature. Two preparations were fed, one unheated and one autoclaved.

RESULTS

The five varieties of beans when fed in the raw state failed to support normal growth, and all were greatly improved by autoclaving (table 1). Chemical determinations of the total sulfur by peroxide fusion indicated that the sulfur content of these varieties did not differ so widely as might have been assumed from a report on their cystine content (Hamilton and Nakamura, '40). Attempts to determine cystine revealed that the Hamilton and Nakamura technique, which involved an acid hydrolysis of NaOH extracts in the presence of large amounts

of nonprotein material, gave unreliable results * (Bailey, '37, and Lugg, '38).

While the percentage of nitrogen absorbed varied somewhat between animals of a given group the average figures were much alike for each of the five varieties studied. The values for the autoclaved beans were consistently higher than those obtained for the raw beans (3.9 to 6.1%). Again, differences between varieties were negligible. With respect to the efficiency of the raw protein to promote growth the Illini variety was somewhat superior. This superiority, however, did not carry over into the autoclaved series.

TABLE 1

A comparison of the nutritive value of the protein of five varieties of soybeans.

VARIETIES OF SOYBEANS	SULFUR CONTENT	BODY WEIGHT (AVERAGE)		NITROGEN ABSORBED		EFFICIENCY OF PROTEIN
		Initial	Final	Range	Average	
Raw	%	gm.	gm.	%	%	
Illini	0.36	56.0	72.8	75.1-86.0	78.6	0.484
Mansoy	0.41	61.3	57.0	70.8-85.3	78.0	0.015
Dunfield	0.36	61.0	64.3	74.1-78.0	76.1	0.119
Virginia Brown	0.38	59.5	53.5	73.8-79.6	77.3	0.201
Mandarin	0.43	58.0	62.3	74.2-78.8	76.1	0.163
Autoclaved						
Illini	...	61.8	164.8	79.7-85.8	83.5	1.700
Mansoy	...	61.2	155.0	80.6-85.1	82.8	1.657
Dunfield	...	58.8	176.0	81.1-83.9	82.2	2.028
Virginia Brown	...	56.5	153.0	76.1-83.7	81.2	1.633
Mandarin	...	60.5	129.3	76.3-86.7	80.3	1.582

Four animals in each group.

Germinated and immature raw beans were distinctly superior to the mature bean. All three, however, were inferior to the corresponding autoclaved preparations. Especially noteworthy is the fact that while the percentage of nitrogen absorbed from the raw preparations is distinctly less than that absorbed from the autoclaved samples the values do not differ greatly. The efficiency of the protein consumed in promoting growth, however, ranged from 0.48 for the mature seed to 1.4 for the germinating seed (table 2). This supports the surmise that the differences between raw and heated mature beans are not due entirely to the improved digestibility of the latter.

* Unpublished data.

TABLE 2

The effect of heat on the nutritive value of Illini soybean protein.

SOYBEANS	BODY WEIGHT (AVERAGE)		NITROGEN ABSORBED	EFFICIENCY OF PROTEIN Grams gain/grams consumed
	Initial	Final		
Raw	gm.	gm.	%	
Mature	56.0	72.8	78.6	0.484
Germinated	58.0	136.2	...	1.504
(3 preparations)	62.4	128.2	...	1.415
	60.2	117.5	75.7	1.299
Immature	60.5	127.2	79.5	1.101
Autoclaved				
Mature	61.8	164.8	83.5	1.700
Germinated	57.3	185.0	83.2	1.903
Immature	60.5	211.5	87.8	2.013

Six animals in each group.

SUMMARY

Five varieties of soybeans including the Illini, Dunfield, Mansoy, Virginia Brown, and Mandarin varieties were studied, heated and unheated, for the nutritive value of their protein. No appreciable varietal differences were observed. All varieties were greatly improved by heating.

The protein of freshly germinated Illini beans was superior in nutritive value to the protein of the unheated mature beans, although the percentage of nitrogen absorbed was not increased.

Immature soybeans likewise supplied protein of higher nutritive value than that of raw mature beans. The protein of germinated as well as of immature soybeans was improved by heating.

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A SHORT METHOD OF CALCULATING THE NUTRITIVE VALUE OF DIETS (*concluded*)

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Food habits of representative sections of the civilian population in this country have been discussed in detail by Stiebeling and Phipard, ('39). In connection with the nutritional evaluation of food provided for the soldier, an opportunity of obtaining an indication of military food habits has presented itself. Since the ration provided for the soldier subsisting on Field Ration A is equivalent roughly to a liberal civilian diet, it is of particular interest to note which foods are the most popular, and the approximate quantities of each which have been planned per day for each man. Furthermore, such information is of particular current interest, since it is an indication of which foods are likely to be made available for the Army.

In addition, the food pattern derived from these quantities of food forms the basis for a short method of nutritional evaluation of food as purchased. The method is based on the classification (Howe, Pritchett and Berryman, '42) of all nutritionally important foods into fifteen food groups, with the subsequent development of caloric, protein, mineral and vitamin values for each group, on the basis of quantitative use of the component items of the group. It is the purpose of this communication to present a discussion of food habits, and their relation to nutritive values for food groups.

The data presented herein have been obtained from records of food planned for the average soldier during the year May 1941 to April 1942, inclusive. The nutritive values for the first 6 months May to October 1941 have been called, for convenience, "summer" values, while those for November 1941 to April 1942, also presented here, are designated as "winter" values. The background of the methods used in computing these values, and of developing group values for use in connection with the short method of nutritional evaluation, has already been discussed (Berryman and Chatfield, '43).

During the period under discussion, quantities of food as shown in table 1 were planned for the soldier (pounds per man per day). Nutritional evaluation of the food indicates that it could be expected to contain approximately the amounts of nutritive components given in table 2.

TABLE 1

Pounds per man per day of foods prescribed for the soldier.

	SPRING-SUMMER MAY 1941-OCT. 1941	FALL-WINTER NOV. 1941-APRIL 1942	AVERAGE
Meats, fish and poultry	.845	.856	.851
Eggs	.178	.151	.165
Milk and milk products (fluid equivs.)	1.085	1.028	1.057
Butter	.093	.091	.092
Fats, other	.085	.079	.082
Grain products (Bread)	.734 (.449)	.662 (.383)	.698 (.357)
Legumes dry and nuts	.066	.054	.060
Sugars and syrups	.340	.301	.321
Vegetables, leafy green and yellow	.420	.441	.431
Tomatoes	.160	.133	.147
Citrus fruits	.210	.190	.200
Potatoes	.654	.697	.676
Vegetables, other	.301	.272	.287
Fruits, other than citrus	.410	.322	.366
Fruits, dried	.034	.033	.034

TABLE 2

Nutritive components per man per day provided by foods in table 1.

	SPRING-SUMMER MAY 1941-OCT. 1941	FALL-WINTER NOV. 1941-APRIL 1942	AVERAGE
Calories	4320	4080	4200
Protein (gm.)	131	125	128
Fat (gm.)	195	189	192
Carbohydrate (gm.)	513	470	492
Calcium (mg.)	1012	960	986
Phosphorus (mg.)	2030	1940	1985
Iron (mg.)	24.5	23.6	24.1
Vitamin A (I.U.)	12900	14000	13450
Thiamine (mg.)	2.98 (2.00) ¹	3.29 (2.25) ¹	3.14 (2.13) ¹
Riboflavin (mg.)	2.50 (2.10) ¹	2.37 (2.00) ¹	2.44 (2.05) ¹
Nicotinic acid (mg.)	26.6 (20.7) ¹	26.9 (2.10) ¹	26.8 (20.9) ¹
Ascorbic acid (mg.)	137 (102) ¹	146 (104) ¹	142 (103) ¹

¹ Represents values after conservative deductions are made for probable losses due to cooking, preparation, etc.

Consideration of the quantities of individual food items planned for use by the soldier shows that certain foods usually predominate within a group. It is these particular items which undoubtedly will continue to be needed for military use within this country, provided they are available at all. Furthermore, the items which predominate have the greatest influence on the nutritive value of the food group involved.

Detailed presentation of the food pattern for the soldier follows in table 3; included are the levels for the two periods under discussion, namely: Spring and Summer 1941, and Fall and Winter 1941-1942. In addition, the values for Fall and Winter of 1942-1943 are presented for comparison purposes. The amounts of the various food items are presented as percentages of the food group, with the quantity of each food group being listed in pounds at the end of the group. "Miscellaneous" comprises those individual foods which were used in small quantities, i.e., lower than the lowest percentage figure listed.

Inspection of the various quantities of food items used indicates that the quantitative food pattern has not changed markedly, with the exception of certain items such as fresh corn, watermelon, cantaloupes, honeydew melon, apples, etc., the use of which obviously varies with the season. Other notable exceptions are boneless beef, which appears at a zero level in the 1943 table, and certain vegetables, the use of which has been decreased as a result of experience with the food habits of the average soldier.

NUTRITIONAL EVALUATION

A method for dietary evaluation based on the food pattern for the 6 months, May to October 1941, has been described by Berryman and Chatfield, '43. For purposes of convenience, the group nutritive values obtained were designated as "summer" values, and it was planned to obtain similar group values for November 1941 to April 1942, to be designated as "winter" values. The basis for the latter would, of course, be the food pattern for those months as presented below;

A study of the "winter" food pattern, and comparison with that of the "summer" indicated, however, that the differences were not as great as had been anticipated. Except for minor variations, which may well be ignored in view of the limits of accuracy of any dietary evaluation based on average nutritive values, foods within the following food groups showed but little deviation in the quantities planned for the two 6-month periods: milk products; fats, other; grain products; legumes dry, and nuts; sugars and syrups. Furthermore, groups containing only one food obviously did not require recalculation. These groups are as

TABLE 3

Pattern of food supply for the United States Army.

FOOD	SPRING AND SUMMER MAY 1941- OCT. 1941	FALL AND WINTER NOV. 1941- APRIL 1942	FALL AND WINTER NOV. 1942- APRIL 1943	FOOD	SPRING AND SUMMER MAY 1941- OCT. 1941	FALL AND WINTER NOV. 1941- APRIL 1942	FALL AND WINTER NOV. 1942- APRIL 1943
	%	%	%		%	%	%
<i>Meats, Fish and Poultry</i>				Potatoes, white	100.	100.	100.
Beef				<i>Pounds/man day</i>	0.65	0.70	0.67
Carcass	18.86	19.35	38.37	Butter	100.	100.	100.
Rounds	8.12	0.50	0.15	<i>Pounds/man day</i>	0.093	0.099	0.08
Boneless	0.08	13.59		Other fats			
Total	25.06	33.44	38.52	Lard or lard sub.	73.79	78.17	74.90
Pork				Mayonnaise	21.84	18.02	21.01
Ham, fresh	1.54	1.78	1.09	Miscellaneous	4.37	3.81	4.09
Boston Butts	1.75	2.20	2.51	<i>Pounds/man day</i>	0.08	0.08	0.07
Chops	0.24	0.04	0.12	<i>Grain Products</i>			
Loin	2.66	5.15	4.36	Bread, issue	60.94	57.84	53.52
Shoulder	0.84	1.40	0.66	Flour, issue	19.58	21.83	27.04
Ham SC	7.48	5.60	7.48	Cereals, dry prepared	4.27	3.59	3.55
Total	14.51	16.17	13.07	Miscellaneous	15.21	16.74	15.89
Veal	4.14	5.26	5.44	<i>Pounds/man day</i>	0.74	0.66	0.67
Bacon	7.67	6.19	6.01	<i>Legumes, dry (beans, peas, etc.)</i>			
Fowl				Navy beans	36.21	42.96	42.80
Chicken	6.81	7.11	7.34	Black eyed peas	7.73	10.56	5.17
Turkey	2.26	2.26	1.80	Lima beans	25.45	23.70	23.06
Duck	0.40	0.65	0.38	Kidney beans	16.06	11.11	15.31
Total	9.47	10.02	9.52	Others	14.55	11.67	13.66
Fish	6.92	6.90	4.46	<i>Pounds/man day</i>	0.07	0.05	0.05
Sausages	6.82	6.72	5.97	<i>Sugars and Syrups</i>			
Miscellaneous	25.41	13.30	17.01	Sugar, granulated	76.02	73.08	71.71
<i>Pounds/man day</i>	0.85	0.86	0.93	Syrup	10.44	10.47	10.03
Eggs, fresh	100.	100.	100.	Jam-preserves	9.95	8.84	6.63
<i>Pounds/man day</i>	0.18	0.15	0.16	Miscellaneous	3.59	7.61	11.63
<i>Milk products (fluid equivalents)</i>				<i>Pounds/man day</i>	0.34	0.30	0.26
Fresh milk	46.21	48.42	46.49	<i>Vegetables other than leafy green or yellow</i>			
Evaporated	34.56	36.10	35.59	Beets, canned and fresh	10.48	12.93	10.94
Cheese	12.24	12.89	14.98	Celery	13.07	15.06	15.59
Ice cream	5.77	2.10	2.20	Corn, canned	11.71	11.79	12.03
Miscellaneous	1.22	0.49	0.74	Corn, fresh	8.98	0.33	0.47
<i>Pounds/man day</i>	1.06	1.03	1.11	Onions	25.62	27.03	25.37
<i>Vegetables, leafy green or yellow (fresh and canned)</i>				Turnip roots	4.62	8.25	6.50
Cabbage	22.11	17.47	15.91	Sauerkraut, canned	5.85	4.94	5.23
Carrots	11.04	12.80	14.11	Miscellaneous	19.67	19.67	23.57
Lettuce	16.07	11.91	10.93	<i>Pounds/man day</i>	0.30	0.27	0.28
Peas	9.57	9.19	7.67	<i>Fruits other than Citrus (fresh and canned)</i>			
Sweet potatoes	8.97	12.82	15.16	Apples, fresh	16.35	31.63	46.55
String beans				Bananas	10.81	19.19	6.93
(canned and fresh)	10.78	9.19	9.17	Peaches, fresh	6.66		0.38
Spinach (canned)	7.54	3.88	1.53	Cantaloupe, fresh	17.61	0.53	0.31
Miscellaneous	13.92	22.74	25.52	Honeydew, fresh			
<i>Pounds/man day</i>	0.42	0.44	0.45	Watermelon, fresh			
Tomatoes, etc.				Apples, canned	11.62	9.43	5.50
Tomatoes, canned	49.66	56.68	48.73	Peaches, canned	8.59	7.53	6.86
Tomatoes, fresh	38.05	21.11	26.03	Pineapple, canned	6.32	6.94	4.13
Tomato juice	3.24	10.11	13.37	Miscellaneous	22.04	24.75	29.34
Tomato catsup	7.86	9.36	10.32	<i>Pounds/man day</i>	0.41	0.32	0.29
Miscellaneous	1.19	2.74	1.55	<i>Fruits, Dried</i>			
<i>Pounds/man day</i>	0.16	0.13	0.14	Apples	2.05	2.75	8.61
<i>Citrus Fruits</i>				Apricots	14.04	16.21	8.01
Grapefruit, fresh	32.76	41.32	37.47	Peaches	16.37	14.68	8.31
Lemons	22.51	8.00	10.52	Prunes	33.92	23.55	27.00
Oranges, fresh	44.21	46.16	49.24	Raisins	33.62	41.90	47.48
Miscellaneous	0.52	4.52	2.77	Miscellaneous	0.0	0.91	0.59
<i>Pounds/man day</i>	0.21	0.19	0.28	<i>Pounds/man day</i>	0.03	0.03	0.03

follows: eggs; fats, butter; potatoes, white. The number of groups remaining closely similar in the distribution of their component items or requiring no change from previous calculation was therefore eight; this left seven food groups which were nutritionally significant and which required further calculation based on the food pattern of the second 6 months.

The tables of weighted "winter" and "summer" nutritive values for the various classes of food are presented in tables 4 and 5 respectively.

It then became of interest to speculate on the possible development of an annual table which might be usable without regard to season. Numerous calculations were made between the accuracy obtained by the long method of calculation (i.e., using individual food values) and the contemplated "annual" table. Furthermore, the accuracy of the latter was compared with that of the winter and summer tables. While it appeared that such a table would give an evaluation which would be reasonably valid in view of the wide possible variation of nutrients in various species of foods, yet a satisfactory degree of accuracy in calculation was not obtainable. This was particularly true in the case of ascorbic acid and Vitamin A, due primarily to seasonal changes in the availability and use of certain fruits and vegetables. It is therefore considered advisable to use separate tables for the winter and summer months (table 4 and table 5), in preference to a single table for year-around use.

DISCUSSION

As stated above, the values for Vitamins A and C in the "summer" and "winter" tables show appreciable differences in some cases. The major reasons for the variations in the two sets of values have been investigated, and are presented below in some detail.

Vitamin A

In so far as Vitamin A is concerned, the chief differences lie in the changing distribution of items in the following groups: leafy green and yellow vegetables, tomato products, and fruits other than citrus. The values for Vitamin A per pound of these food groups are as follows (taken from tables 4 and 5).

Vegetables, leafy green and yellow

May–October 1941
14,750 I.U. per lb.

November 1941–April 1942
16,100 I.U. per lb.

The one item contributing chiefly to this change is sweet potatoes, which in terms of percentage, showed an increase of 8.97 to 13.52%.

TABLE 4
Short method of computing nutritive value of ration — (Winter).
Nutrients per pound of food groups "as purchased" (based on food prescribed for U. S. Army Nov. 1941–April 1942).

GROUP	CALORIES	PROTEIN	FAT	C(OH)	Ca	P	Fe	VITAMIN A	THIAMINE	RIBO-FLAVIN	NICOTINIC ACID	ASCORBIC ACID
	gm.	gm	gm	gm	mg	mg.	mg	I.U.	mg.	mg.	mg.	mg.
Meats, fish and poultry	1115	66	95	2	42	728	10.4	117 ¹	1.93 ²	.79 ³	19.9	4
Eggs, fresh	635	52	46	3	218	848	10.9	4040	.56	1.45	.2	0
Milk products (equivs.)	360	16	21	25	556	427	.9	1020	.11	.74	.4	4
Fats, butter	3320	3	367	2	73	73	.9	14970	.01	.04	.5	0
Fats, other	3900	1	432	3	2	9	.1	120	.01	.01	.2	0
Grain products ⁴	1360	40	14	270	160	482	9.0 ²	20	1.12 ²	.83 ²	11.2 ²	0
Legumes, dry and nuts	1630	97	15	276	538	1968	40.7	10	2.40	1.33	12.5	0
Sugars and syrups	1680	1	0	420	37	16	2.2	0	.00	.00	0.0	0
Vegetables, leafy green and yellow	173	6	1	35	157	158	3.4	16100	.31	.26	2.7	116
Roots	138	5	1	29	64	179	2.5	3335	.36	.20	2.0	69
Citrus fruits	147	2	47	33	84	66	1.1	96	.26	.08	1.6	121
Potatoes, white	330	8	0	73	30	186	2.8	150	.34	.19	4.5	46
Vegetables, other	190	6	1	39	116	175	2.1	155	.13	.23	1.0	64
Fruits, other than citrus, fresh and canned	330	2	1	78	34	54	1.4	1050	.09	.19	1.5	18
Fruits, dried	1300	13	2	307	253	453	17.7	8130	.47	.40	6.8	0

¹ When liver is used add 125,000 I.U. Vitamin A and 14.56 mg. of riboflavin per pound of liver.

² Use this figure only when the combined weight of the lean pork cuts (viz., fresh and cured ham, pork loin and chops, Boston butt, and shoulder) is 15% or more of the weight of the meat group. When the combined weight is less than 15%, compute the thiamine thus: (wt., in lbs., of lean pork cuts \times 5.9) plus (wt., in lbs., of all other meat \times 1.0). This gives the quantity of thiamine in milligrams in the entire group.

³ When enriched flour and bread are not used, count thiamine in the entire group as only 0.59 mg. per pound, riboflavin 0.29 mg. per pound, nicotinic acid 4.2 mg. per pound, and iron 5.5 mg. per pound (based on straight flour); no changes need be made in other nutrients.

⁴ Values based on new enrichment levels effective October 1, 1943.

TABLE 5
*Short method of computing nutritive value of ration (Summer).
 Nutrients per pound of food groups "as purchased" (based on food prescribed for U. S. Army May-October 1941).*

GROUP	CALORIES	PROTEIN	FAT	C(OH)	Ca	P	Fe	VITAMIN A	THIAMINE	RIBO-FLAVIN	NIACOTINIC ACID	ASCORBIC ACID
	gm.	gm.	gm.	gm.	mg.	mg.	mg.	I.U.	mg.	mg.	mg.	mg.
Meats, fish and poultry	1120	65	95	2	41	719	9.8	120 ²	1.87 ²	.77 ¹	19.4	3
Eggs, fresh	635	52	46	3	218	848	10.9	4040	.56	1.45	.2	0
Milk products (equivs.)	360	16	21	25	556	427	.9	1020	.11	.74	.4	4
Fats, butter	3320	3	367	2	73	73	.9	14970	.01	.04	.5	0
Fats, other	3900	1	432	3	2	9	.1	120	.01	.01	.2	0
Grain products ⁴	1360	40	14	270	160	482	9.0 ²	20	1.12 ²	.83 ²	11.2	0
Legumes, dry and nuts	1630	97	15	276	538	1968	40.7	10	2.40	1.33	12.5	0
Sugars and syrups	1680	1	0	420	37	16	2.2	0	.00	.00	.0	0
Vegetables, leafy green and yellow	150	7	1	30	145	152	3.5	14750	.29	.24	.27	91
Tomatoes	130	5	1	26	61	160	2.6	3860	.36	.20	2.2	78
Citrus fruits	140	2	1	32	91	66	1.2	90	.23	.07	1.5	118
Potatoes, white	330	8	0	73	30	186	2.8	150	.34	.19	4.5	46
Vegetables, other	180 ¹	6	1	37	102	173	1.9	180	.12	.22	.9	49
Fruits, other than citrus, fresh and canned	290	2	1	69	34	51	1.3	1940	.09	.17	1.4	24
Fruits, dried	1280	12	2	302	245	452	18.0	8320	.47	.60	6.6	0

¹ When liver is used add 125,000 I.U. Vitamin A and 14.56 mg. of riboflavin per pound of liver.

² Use this figure only when the combined weight of the lean pork cuts (viz., fresh and cured ham, pork loin and chops, Boston butt, and shoulder) is 15% or more of the weight of the meat group. When the combined weight is less than 15% compute the thiamine thus: (wt., in lbs., of lean pork cuts \times 5.9) plus (wt., in lbs., of all other meat \times 1.0). This gives the quantity of thiamine in milligrams in the entire group.

³ When enriched flour and bread are not used, count thiamine in the entire group as only 0.59 mg. per pound, riboflavin 0.29 mg. per pound, nicotinic acid 4.2 mg. per pound, and iron 5.5 mg. per pound (based on "straight" flour); no changes need be made in other nutrients.

⁴ Values based on new enrichment levels effective October 1, 1943.

In terms of International Units per pound, this is equivalent to an increase of approximately 820, or more than half of the change in the value per pound for the group. Other cumulative increases in the quantities of such items as carrots, squash, pumpkin, turnip greens, etc., with the concomitant decrease in the use of items relatively dilute with regard to Vitamin A (i.e.; cabbage, lettuce, etc.), resulted in further increase in the overall Vitamin A value of the group for the winter months.

Tomatoes

May–October 1941
3860 I.U. per lb.

November 1941–April 1942
3335 I.U. per lb.

The drop in the use of fresh tomatoes was primarily responsible for these changes. A drop of approximately 17% in the use of this item, is equivalent to a change in terms of I.U. per pound of approximately 1400. Part of this change was offset by the increased percentage use of canned tomato products; it was not entirely offset, however, because of the lower Vitamin A value of the latter. The net result was, therefore, a drop of about 500 I.U. per pound in the winter value.

Fruits other than citrus

May–October 1941
1940 I.U. per lb.

November 1941–April 1942
1050 I.U. per lb.

The items responsible for this change are the various types of melons (cantaloupe, honeydew) and fresh peaches, the use of which during the winter months decreased concurrently with an increase in the use of Vitamin A dilute items such as apples, bananas, etc.

Vitamin C

The changes in Vitamin C values per pound were greatest in the “leafy green or yellow vegetable” and “other vegetables” groups.

Vegetables, leafy green and yellow

May–October 1941
91 mg. per lb.

November 1941–April 1942
116 mg. per lb.

The items responsible for the change in Vitamin C values per pound of leafy green-yellow vegetables are not, as might be first considered, the change in sweet potatoes, since the increased use of this item was approximately offset with regard to Vitamin C by a decrease in the use of cabbage. Rather the small but cumulative increases in Vitamin C rich items such as turnip greens, spinach, broccoli, green peppers, etc., became of importance when considered together with the simultaneous

decrease in the use of lettuce and other items which are less rich in the vitamin.

Vegetables other than leafy green and yellow.

May–October 1941

49 mg. per lb.

November 1941–April 1942

64 mg. per lb.

The increased use of the following, together with a decreased use of fresh corn and other Vitamin C dilute items were responsible for the change in the value per pound for the group: green lima beans; cauliflower; green onions; rutabaga; and turnips.

Boneless versus carcass beef

The question is frequently raised as to what correction should be made when large quantities of boneless beef are used. Since the use of boneless beef during 1943 has been negligible within this country, the matter probably does not warrant detailed discussion. However, the answer to be given to such a question is that usually no correction is needed. One example will serve to illustrate the point. If all of the quantity of boneless beef used during November 1941 to April 1942 were converted to a carcass beef equivalent by use of the ratio 1:1.4, the result would be a change from .1163 pounds per man per day of boneless beef to .1628 pounds per man per day of carcass beef equivalent. The change in percentage contribution of carcass beef would be from 19.35 to 38.3, with the boneless percentage, of course, being zero. (This situation is analogous to that observed for the same items during November 1942 to April 1943). The error (per pound of “meats, fish, and poultry”) involved by using the values as they now stand would be as follows:

CALS.	PROT.	FAT	C(OH)	Ca	P	Fe	A	THIAMINE	RIBO- FLAVIN	NICOTINIC ACID	ACID
	gm.	gm.	gm.	mg	mg	mg.	I.U.	mg.	mg.	mg.	mg.
32	2.2	2.2	0	1.2	23.4	.33	0	.02	.02	0.6	0

Such error in calculation is obviously of the slightest magnitude, and not within the province of the intended purpose of a method of evaluating the approximate nutritive value of the diet.

SUMMARY

The food pattern planned for the soldier subsisting on Field Ration A during the year May 1941 to April 1942 has been described. Seasonal tables of nutritive values based on this food pattern have been presented as a rapid means of calculating the probable nutritive value of food

supplied for Field Ration A, the rough equivalent of a liberal civilian diet. Reference is made to a previous publication for the detailed background of such tables, and for the degrees of accuracy made possible by their use.

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HUMAN RESPIRATORY QUOTIENTS IN RELATION TO ALVEOLAR CARBON DIOXIDE AND BLOOD LACTIC ACID AFTER INGESTION OF GLUCOSE, FRUCTOSE, OR GALACTOSE¹

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The changes in the respiratory quotient after the ingestion of hexoses have been studied by many investigators, and attempts have been made to ascertain whether the rises in respiratory quotient are ascribable to a combustion of the sugar, to a formation of fat from the sugar, to a change in the acid-base condition of the blood with a resultant displacement of carbon dioxide, or to a combination of these three processes. Nevertheless it still remains an unsettled question whether there are any real differences in the effects on the respiratory quotient of the ingestion of the three principal hexoses, glucose, fructose, and galactose. The object of the present research was to determine the respiratory quotients of human subjects after ingestion of standard amounts of these three hexoses taken separately, and periodically to ascertain the lactic acid content of the blood. Simultaneously with the measurement of the respiratory exchange, samples of alveolar air were drawn for analysis of the carbon dioxide content.

While our research was in progress, Bachmann and Haldi ('37) reported the results of a study in which two of these sugars were given to three male adults and determinations made of the carbon dioxide and the lactic acid in blood. After the ingestion of 50 gm. of glucose, there was no increase in the lactic acid and no decrease in the carbon-dioxide content of the blood, and they concluded that the respiratory quotients obtained within 30 minutes after the ingestion of glucose were true metabolic quotients. The same quantity of fructose led to an in-

¹ A preliminary report of this study was presented before the American Institute of Nutrition, Memphis, Tennessee, April 21, 1937 (Carpenter, Bensley, Dill, and Edwards, '37).

² Died December 14, 1937.

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crease in the lactic acid and a decrease in the carbon dioxide content of the blood. They believe that the respiratory quotients after ingestion of fructose are not true metabolic quotients. When correction was made for the changes in lactic acid and carbon dioxide in the blood, the quotients obtained within 30 to 45 minutes after ingestion of fructose were considerably higher than those obtained in a corresponding period after ingestion of glucose, and they were led to the conclusion that relatively more carbohydrate was burned after the ingestion of fructose or that this sugar was transformed into fat.

Stöhr ('33) found that 0.2 gm. of glucose per 100 gm. of body weight, given to rats that had been 24 hours without food, resulted in a lowering of the alkali reserve after 15 to 30 minutes, but that this reserve returned to normal after 1 hour. Fructose had no effect in this respect. Sterkin and Wengerowa ('34) observed a marked rise in the lactic acid of the blood of dogs after oral ingestion of 2 gm. of fructose per kilogram of body weight, but after glucose only variations in either direction. Lactose produced no change. A study of Blatherwick, Bradshaw, Ewing, and Sawyer ('40) showed that, 1 hour after ingestion by stomach tube, fructose produced with rats more than a 50% increase in the lactic acid content of liver and muscles as compared with the results when glucose was given. These findings they consider as evidence that lactic acid was largely responsible for the higher respiratory quotients after fructose. Sachs, Sternfeld, and Kraus ('42) found no rise in the lactic acid of the blood after oral ingestion of 50 gm. of fructose in two boys who showed essential fructosuria, whereas with two normal controls they found marked rises within 30 and 65 minutes after ingestion.

EXPERIMENTAL

The subjects of the experiments were two men, H. C. W. (age, 21 years; height, 176 cm.; weight, 69.4 kg.) and W. C. (age, 19 years; height, 170 cm.; weight, 70.3 kg.). The subject came to the laboratory in the post-absorptive condition and sat in a comfortable chair for half an hour. He then began breathing in the respiration apparatus, and during the next hour the respiratory exchange was measured in three consecutive 15-minute periods. Alveolar air samples were taken simultaneously at approximately 5-minute intervals. After these three basal periods, the subject released the mouthpiece of the respiration apparatus, and a sample of venous blood was drawn with the subject sitting. The dose of sugar for the day was then given, following the ingestion of which the subject continued breathing in the respiration apparatus for

two or three more consecutive 15-minute periods, and alveolar air samples were again taken. Another blood sample was drawn, two or three more 15-minute periods were run, and so on until a total of approximately 4 hours had elapsed after the dose was given. The amount of sugar given was 50, 75, or in most instances 100 gm. This was taken in 310 ml. of water, after which the subject drank the small amount of water (25 ml.) used to wash the beaker and tube. In no-dose control experiments the same routine of period measurements was carried out.

The respiratory exchange was determined by the use of the mouth-piece and open-circuit apparatus of Carpenter and Fox ('31), and the alveolar air samples were obtained by the technique of Carpenter and Lee ('33a). All samples of expired air and of alveolar air were analyzed in duplicate by means of the portable Haldane gas-analysis apparatus. The blood samples were analyzed for lactic acid by the method of Edwards ('38).

RESULTS

Alveolar carbon dioxide

The results of the alveolar carbon dioxide determinations are given in table 1, expressed as the average partial pressures in millimeters noted in the preliminary 45 minutes of observation before sugar ingestion and in the several groups of periods after the dose. In the control experiments, the alveolar carbon dioxide of H. C. W. was slightly lower in general throughout the series than in the preliminary 45 minutes. With W. C. there was but little difference in the alveolar carbon dioxide, although there was a slight tendency for it to fall in the course of the 4 hours. In the experiments with glucose, there was an average drop with H. C. W. from 42.0 to 41.0 mm. at the end of the experiments, but with W. C. there was practically no change in the entire series. In the fructose experiments, there was little change with H. C. W. and with W. C. a drop of about 1 mm. In the galactose experiments with H. C. W. there was at first a slight rise, then a return to the pre-ingestion level (average, 42.8 mm.), and thereafter throughout the rest of the experiment a decided drop, the final average value for the two experiments being 40.2 mm. Similarly with W. C., there was a drop from the average initial level of 39.4 mm. to 37.9 mm. at the end of the experiments. With H. C. W. the lowest alveolar carbon dioxide came at the end of the galactose experiments, whereas with W. C. it occurred in the period from 135 to 180 minutes after the dose. These findings accord with the results obtained by one of us (Carpenter, '37; Carpenter and Lee, '33b) in pre-

vious studies, in which no significant change was noted in the alveolar carbon dioxide of a trained human subject after ingestion of 25 gm. of fructose or glucose, whereas a marked decrease was observed after ingestion of 25 gm. of galactose. In the earlier experiments the peak of the change in alveolar carbon dioxide was reached much earlier than in the present series of experiments, in which the dose was 100 gm.

TABLE 1

Average alveolar carbon dioxide before and after ingestion of glucose, fructose, or galactose (millimeters partial pressure).

SUBJECT AND TYPE OF EXPT.	DATE (1936)	PRELIM 45 MIN.	MINUTES AFTER DOSE ¹					
			0-45	45-90	90-135	135-180	180-240	
H. C. W.	Control	April 28	42.6	41.1	40.9	40.8	39.8	40.5
		May 7	43.7	42.3	41.8	42.0	41.8	41.9
		May 21	42.7	43.0	42.6	42.4	42.8	43.0
		June 5	41.2	40.9	41.0	40.7	40.6	40.6
	Glucose	May 19	42.4	42.0	41.5	41.0	41.2	40.7
		June 1	41.6	42.3	42.4	41.7	41.3	41.3
	Fructose	May 14	42.5	42.5	41.8	41.3	41.5	42.0
		May 28 ²	41.9	42.2	41.1	41.2	41.6	41.7
	Galactose	May 12	42.1	43.1	41.3	41.2	40.6	39.1
		May 23	43.5	44.0	43.6	42.3	41.5	41.2
W. C.	Control	May 26 ³	(38.9)	(37.2)	(37.4)	(34.1)	(35.8)	(36.0)
		June 3	36.8	36.5	36.0	36.1	35.7	35.2
		June 18	37.7	38.2	38.5	38.1	37.4	38.0
		July 3	40.2	40.0	40.2	39.8	40.0	39.5
	Glucose	June 8	36.2	36.3	35.9	36.3	35.6	35.2
		June 19	37.4	37.6	37.9	38.4	37.6	38.4
	Fructose	June 12 ⁴	37.1	36.5	36.2	37.1	37.5	37.2
		June 24 ⁴	39.5	38.4	37.4	37.8	38.7	38.7
		June 26	40.7	40.3	38.6	38.9	38.7	39.5
		June 29	41.8	41.6	40.1	39.9	39.9	39.6
	Galactose	June 10	37.3	37.0	36.2	36.0	35.8	36.1
		June 22	39.7	39.2	38.4	38.7	37.6	38.4
		July 1	41.1	41.6	39.7	39.1	38.4	39.1

¹ 100 gm. of sugar, unless otherwise noted.

² 50 gm. of sugar.

³ This was W. C.'s first experience in having alveolar-air samples taken.

⁴ 75 gm. of sugar.

Blood lactic acid

The data regarding lactic acid in the blood are given in table 2. In the control series the most striking changes occurred in the first experiment with H. C. W., on April 28. The procedure for taking the blood samples in this experiment was different from that in any of the following experiments because, instead of remaining seated, the subject rose from the chair and reclined on a couch before the sample was taken.

TABLE 2

*Lactic acid in blood before and after ingestion of glucose, fructose, or galactose
(milligrams per 100 ml.).*

SUBJECT AND TYPE OF EXPT.	DATE (1936)	BASAL	MINUTES AFTER DOSE ¹				
			45 ±	90 ±	135 ±	180 ±	240 ±
H. C. W. Control	April 28	(9.7)	(10.5)	(9.7)	...	(11.2)	...
	May 7	7.4	6.7	6.7	6.7	6.7	...
	May 21	8.2	7.5	6.0	6.7	6.0	...
	June 5	6.7	6.7	7.5	8.2	7.5	7.5
Glucose	May 19	8.2	11.2	9.0	9.7	9.7	...
	June 1	7.5	11.2	9.0	9.0	12.7	8.2
Fructose	May 14	9.0	21.7	19.5	15.7	10.5	...
	May 28 ²	6.7	15.7	10.5	9.0	7.5	6.7
Galactose	May 12	6.7	12.0	15.0	12.0	12.0	...
	May 23	6.7	11.2	10.5	10.5	11.2	9.0
W. C. Control	May 26	11.2	8.2	6.7	6.7	7.5	...
	June 3	6.0	6.0	9.0	9.0	7.5	6.7
	June 18	7.5	8.2	6.7	7.5	6.7	7.5
	July 3	8.2	7.5	7.5	6.7	6.0	6.7
Glucose	June 8	6.7	10.5	9.7	8.2	8.2	8.2
	June 19	8.2	11.2	11.2	10.5	10.5	9.0
Fructose	June 12 ³	7.5	15.7	16.5	13.5	9.0	8.2
	June 24 ³	8.2	15.0	15.0	12.7	9.7	8.2
	June 26	8.2	19.5	19.5	18.7	12.0	7.5
	June 29	8.2	15.7	19.5	15.7	12.0	7.5
Galactose	June 10	7.5	15.0	15.0	12.7	10.5	7.5
	June 22	7.5	21.0	16.5	17.2	9.0	6.7
	July 1	8.3	15.0	15.0	14.2	9.7	6.0

¹ 100 gm. of sugar, unless otherwise noted.

² 50 gm.

³ 75 gm.

This muscular movement was great enough so that it not only raised the general level of the lactic acid in the blood of this subject in this particular experiment compared with the levels noted with the same subject in the other control experiments, but it also produced irregularity in the results, as the range in the values is from 9.7 to 11.2 mg. per 100 ml. In the other control experiments with this subject there is but little irregularity in the values on any individual day, and in general the values on the different days agree fairly well with each other. In the case of W. C. the first blood sample he had ever had taken was that on May 26th. Therefore the higher value in the first sample on this day may be ascribable to greater activity and the slight excitement coincident with the procedure of taking the sample. The subsequent values on that day are not irregular, and for the most part the range in the values on the other 3 days is not wide, with the exception of the two values of 9.0 mg. on June 3rd. The cause for these being higher than the rest of the values is not known.

In the glucose experiments there is a decided rise in the lactic acid values within 45 minutes after the dose. This does not agree with the results of Bachmann and Haldi ('37), who found no change in the blood lactic acid after 50 gm. of glucose. However, as the rise with our subjects was so large and this large rise was found in all four experiments, there is no doubt about its validity. With H. C. W. the values after the forty-fifth minute show little real indication of a rise, with the exception on June 1st of the value of 12.7 mg. in the periods from 135 to 180 minutes after the sugar. With W. C. on June 19th, however, the values remain higher than the pre-ingestion level throughout the experiments. In the fructose series immediately after the dose was given, whether the amount was 50, 75, or 100 gm., there was a marked rise in the lactic acid content of the blood. With H. C. W. on May 14th, the blood lactic acid after 100 gm. of fructose remained higher than the pre-ingestion level throughout the experiment. On May 28th, however, the rise with the same subject after 50 gm. was not so long and was entirely over at the end of 3 hours. With W. C. doses of 50 and 75 gm. caused a rise in the blood lactic acid for 135 minutes, whereas doses of 100 gm. caused a rise for 3 hours. In all four experiments with W. C. the lactic acid at the end of 4 hours had reached the pre-ingestion level. In all the galactose experiments there is a rise in the values immediately after ingestion of the sugar. In both experiments with H. C. W. the lactic acid remained above the pre-ingestion level for at least 3 hours. With W. C. the rise was even greater than that noted with H. C. W., and the lactic acid remained at a higher level until the end of 135 minutes. At 180 minutes

it had dropped practically to the pre-ingestion level, and at 240 minutes it had returned to the pre-ingestion level in all three experiments.

Corrections in respiratory quotients necessitated by changes in alveolar carbon dioxide and blood lactic acid

As these sugars all had definite effects, particularly upon the blood lactic acid and in the case of galactose upon the alveolar carbon dioxide, it became necessary to calculate the effect of the changes in lactic acid and alveolar carbon dioxide upon the respiratory exchange, especially the respiratory quotient. The calculation was made by correcting the carbon dioxide elimination for the change in alveolar carbon dioxide and for the theoretical change produced upon the carbon dioxide content of the blood by the rise or fall of the lactic acid content of the blood. In this calculation it was assumed: (1) that the production and removal of lactic acid are associated with equivalent changes in the carbon dioxide dissociation curve, that is, that a change of 1 millimole in the carbon dioxide combining power of blood is caused by a change in lactic acid concentration of 1 millimole or 9 mg. per 100 ml. — in other words, an increase of 1 mg. % in lactic acid concentration will reduce the carbon dioxide curve 0.247 volume %; (2) that the change in alveolar carbon dioxide tension of itself reflects accurately the change in arterial carbon dioxide tension (Bock and Field, '24); (3) that the change in the carbon dioxide content of the blood water equals the change in the carbon dioxide content of the body water (Irving, Foster, and Ferguson, '32); and (4) that the water of the blood equals 840 ml. per liter and the water of the body is equivalent to 50 liters. Hence a change of 1 volume % in the blood is equal to 1.19 ml. of carbon dioxide per 100 ml. of blood water, or a total of 595 ml. in the body.

The magnitude of the corrections necessary in the respiratory quotients according to calculations based on these assumptions is shown in table 3. In the control experiments lasting a total of 5 hours, there is little or no change in the respiratory quotient that may be ascribed to a change in the lactic acid content of the blood or to a change in the alveolar carbon dioxide, provided the experiments were carried out with extreme care so far as the activity of the subject was concerned. In the glucose experiments the largest correction would be expected in the first group of periods after the dose. With H. C. W. this is so on May 19th, the correction being — 0.04. On June 1st, however, the maximum correction occurs in the period from 135 to 180 minutes. With W. C. there is little or no really significant correction in the quotient for the

entire series, the maximum being ± 0.03 . In the fructose experiments the corrections are somewhat larger. With H. C. W. the maximum corrections in the two experiments are -0.10 and -0.14 in the first group of periods from 0 to 45 minutes. There is also a significant correction in the reverse direction on May 14th, in the period from 135 to 180 minutes. With W. C. the corrections in the first group of periods vary from -0.07 to -0.15 , and corrections of the order of ± 0.03 to ± 0.06 are found in the following periods, the signs changing from minus to plus in the groups of periods from 90 to 135 minutes and thereafter. In the

TABLE 3

Corrections in respiratory quotients based on changes in alveolar carbon dioxide and lactic acid.

SUBJECT AND TYPE OF EXPT.	DATE (1936)	MINUTES AFTER DOSE ¹					
		0-45	45-90	90-135	135-180	180-240	
H. C. W.	Control	April 28	- 0.04	+ 0.01	± 0.00	- 0.04	+ 0.02
		May 7	- 0.02	- 0.01	+ 0.01	- 0.01	± 0.00
		May 21	+ 0.02	+ 0.01	- 0.02	+ 0.02	- 0.01
		June 5	± 0.00	± 0.00	- 0.01	+ 0.02	± 0.00
	Glucose	May 19	- 0.04	+ 0.01	- 0.01	+ 0.01	± 0.00
		June 1	- 0.02	+ 0.03	- 0.02	- 0.05	+ 0.03
	Fructose	May 14	- 0.14	+ 0.01	+ 0.04	+ 0.06	+ 0.03
		May 28 ²	- 0.10	+ 0.04	+ 0.01	+ 0.03	± 0.00
	Galactose	May 12	- 0.04	- 0.07	+ 0.03	- 0.01	+ 0.02
		May 23	- 0.04	± 0.00	- 0.03	- 0.03	+ 0.02
W. C.	Control	May 26	± 0.00	+ 0.02	- 0.06	+ 0.03	± 0.00
		June 3	- 0.01	- 0.04	± 0.00	± 0.00	± 0.00
		June 18	± 0.00	- 0.02	- 0.02	- 0.01	± 0.00
		July 3	± 0.01	+ 0.01	± 0.00	+ 0.01	- 0.01
	Glucose	June 8	- 0.03	± 0.00	+ 0.03	- 0.01	± 0.00
		June 19	- 0.02	± 0.00	+ 0.02	- 0.02	+ 0.03
	Fructose	June 12 ²	- 0.11	- 0.01	+ 0.05	+ 0.06	+ 0.01
		June 24 ²	- 0.11	- 0.02	+ 0.04	+ 0.05	+ 0.01
		June 26	- 0.15	- 0.03	+ 0.01	+ 0.06	+ 0.06
		June 29	- 0.07	- 0.06	+ 0.04	+ 0.04	+ 0.03
	Galactose	June 10	- 0.09	- 0.02	+ 0.02	+ 0.02	+ 0.02
		June 22	- 0.16	+ 0.02	- 0.01	+ 0.07	+ 0.04
		July 1	- 0.08	- 0.04	± 0.00	+ 0.04	+ 0.04

¹ 100 gm. of sugar unless otherwise noted.

² 50 gm.

³ 75 gm.

first two groups of periods the corrections are necessitated by increases in the lactic acid content of the blood, whereas in the remaining three groups they are ascribable to decreases in the lactic acid content, in which equilibrium in blood lactic acid is regained. In the galactose series with H. C. W. there is a correction of -0.07 on May 12th, but this is the largest correction in any of the groups of periods with this subject. With W. C. the corrections in the group of periods after galactose, 0 to 45 minutes, are larger than in the succeeding periods. Thereafter little, if any, correction is necessary except on June 22nd, where there is a correction of $+0.07$ in the group of periods from 135 to 180 minutes. The greatest corrections in the respiratory quotient ascribable either to lactic acid formation or to changes in alveolar carbon dioxide are those in the fructose experiments.

Net changes in respiratory quotients ascribable to sugar ingestion

When the respiratory quotients are thus corrected for the changes in the carbon dioxide and lactic acid contents of the blood, the resultant net changes in the quotients ascribable to the ingestion of the sugars (when the quotients after sugar ingestion are compared with the basal or pre-ingestion levels) are as shown in table 4. In the glucose series in the first group of periods after ingestion of glucose there is, on the average, little or no change ascribable to the sugar. In the second group of periods there is an average change of $+0.08$ and in the third group, of $+0.11$. In the last two groups of periods the average changes are the same with both subjects. The maximum increase in respiratory quotient after glucose ingestion is apparently reached in the periods between 90 and 135 minutes, but in no case is there a return to the pre-ingestion level in 4 hours. With fructose the course of the increases in the respiratory quotient is somewhat similar to that with glucose, except that beginning with the second group of periods, 45 to 90 minutes, the increases are larger than those found with glucose. In other words, after all the corrections necessitated by changes in the carbon dioxide and lactic acid contents of the blood are taken into account, the ingestion of fructose actually produced a greater increase in the respiratory quotient than did the ingestion of glucose. This is particularly true in the periods from 90 to 180 minutes. With galactose the average changes are almost the same as those found with glucose, although the agreement between the two subjects is not so good with galactose.

In general, the greatest increases in the respiratory quotient are after fructose ingestion. This implies that there is a more rapid combustion of carbohydrates after fructose than after glucose or galactose

ingestion. The respiratory quotients considered in table 4 are the total quotients. Some of them approach or equal unity, and if corrections are made for protein combustion in the calculations, the non-protein quotients in these cases will be over 1.00. This applies particularly to those after fructose and after galactose but rarely after glucose ingestion. Respiratory quotients over 1.00 are interpreted as indicative of formation of fat from carbohydrates. It would seem from the increases in the

TABLE 4
Net changes in respiratory quotients ascribable to sugar ingestion.

SUBJECT AND TYPE OF EXPT.	DATE (1936)	BASAL R. Q.	MINUTES AFTER DOSE ¹				
			0-45	45-90	90-135	135-180	180-240
H. C. W.							
	Glucose						
	May 19	0.80	- 0.03	+ 0.09	+ 0.06	+ 0.11	+ 0.09
	June 1	0.75	+ 0.02	+ 0.09	+ 0.12	+ 0.07	+ 0.03
	Fructose						
	May 14	0.84	- 0.01	+ 0.10	+ 0.15	+ 0.12	+ 0.05
	May 28 ²	0.77	+ 0.03	+ 0.12	+ 0.12	+ 0.09	+ 0.02
	Galactose						
	May 12	0.78	+ 0.02	+ 0.03	+ 0.12	+ 0.08	+ 0.08
	May 23	0.78	+ 0.02	+ 0.06	+ 0.07	+ 0.07	+ 0.08
	W. C.						
	Glucose						
	June 8	0.82	- 0.01	+ 0.08	+ 0.16	+ 0.11	+ 0.06
	June 19	0.87	- 0.04	+ 0.06	+ 0.10	+ 0.07	+ 0.06
	Fructose						
	June 12 ²	0.83	+ 0.05	+ 0.08	+ 0.07	+ 0.09	- 0.01
	June 24 ²	0.85	+ 0.02	+ 0.12	+ 0.15	+ 0.02	+ 0.02
	June 26	0.81	- 0.01	+ 0.14	+ 0.16	+ 0.16	+ 0.05
	June 29	0.77	- 0.06	+ 0.01	+ 0.13	+ 0.10	+ 0.03
	Galactose						
	June 10	0.83	+ 0.04	+ 0.12	+ 0.14	+ 0.11	+ 0.04
	June 22	0.83	- 0.03	+ 0.16	+ 0.13	+ 0.16	+ 0.04
	July 1	0.84	- 0.01	+ 0.10	+ 0.08	+ 0.11	+ 0.02

¹ 100 gm. of sugar unless otherwise noted.

² 50 gm. of sugar.

³ 75 gm. of sugar.

respiratory quotient over basal that the order of possibility in this respect is fructose, galactose, and glucose. Therefore, these high respiratory quotients may result from the conversion of sugar to fat as well as from increased combustion.

SUMMARY

The effects of the ingestion of 50, 75, or 100 gm. of glucose, fructose, or galactose on the alveolar carbon dioxide, the blood lactic acid, and

the respiratory quotient were studied over a period of 4 hours with two normal men. In comparison with control experiments, the alveolar carbon dioxide showed a significant change (decrease) only in the galactose experiments. The blood lactic acid increased after all three sugars, the increase being most marked and lasting longest after fructose. The respiratory exchange measurements were corrected for the effects of these changes in blood lactic acid and alveolar carbon dioxide, and the resultant net increases in the respiratory quotients ascribable to the ingestion of the sugars were calculated. After glucose the maximum net increase in quotient occurred within 90 to 135 minutes, and there was no return to the pre-ingestion level in 4 hours. After galactose the net increases were about the same as after glucose. The greatest net increases in the respiratory quotient were produced by the ingestion of fructose. The differences in the rises in quotient indicate differences in the rapidity of combustion of the sugar or differences in the rate at which sugars may be transformed into fat.

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THE THIAMINE REQUIREMENT OF PIGS AS RELATED TO THE FAT CONTENT OF THE DIET¹

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TWO FIGURES

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Data have been presented in a previous article by Van Etten, Ellis, and Madsen ('40) which indicate that young pigs of 1 to 3 months' age require 106 to 120 μ g. of thiamine (vitamin B₁) per 100 gm. of carbohydrate and protein for normal growth. On a body-weight basis and for the diet used, these figures corresponded roughly to 37 μ g. of thiamine per day per kilogram of body weight. This value, which corresponds to approximately 1.7 mg. per 100 pounds of body weight, is somewhat higher than the figure of 1 mg. given by Hughes ('41) who worked with pigs beyond the usual weaning age. The earlier study was based on the use of a diet which contained approximately 10% of fat. The results were further interpreted to indicate that the thiamine requirement of the pig is directly related to the carbohydrate-protein intake.

Such a relationship has been shown in the rat by Evans and Lepkovsky ('29) and in the dog by Arnold and Elvehjem ('39).

Accordingly, additional work was undertaken to establish the relationship of thiamine intake to that of dietary fat and to determine more definitely the thiamine requirements on the basis of carbohydrate-protein consumption. In addition, information was sought on the physiological and pathological response of pigs on different levels of thiamine including storage of the vitamin in the muscle tissue.

PROCEDURE

The role of dietary fat was studied at three levels, namely, approximately 2, 11, and 28%. The first one was selected as representing a

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typical low level of fat such as is sometimes fed to pigs on the farm and at the same time supplying the necessary essential fat acids. The high level approximates the fat content in the total solids of sow's milk and the intermediate level, that of the mixture of grain feed and sow's milk consumed by suckling pigs from the age of 3 weeks to weaning. The diets were of the type used in the earlier work of Van Etten, Ellis and Madsen ('40) and were prepared from constituents which had been treated to remove or inactivate the thiamine without materially reducing the content of the other vitamin factors of the vitamin B complex. Rat assays on the diets have confirmed this premise. The composition of the diets is shown in table 1.

TABLE 1
Composition and comparative feeding levels of the diets.

ITEM	COMPOSITION AND FEEDING LEVEL OF DIETS		
	Low-fat	Medium-fat	High-fat
	%	%	%
Casein, extracted	22.3	25.0	30.3
Dried whey, SO ₂ treated	10.7	12.0	14.5
Dried liver, SO ₂ treated	6.7	7.5	9.1
Salt mixture	3.1	3.5	4.2
Agar	1.8	2.0	2.1
Lard	1.0	10.0	28.0
Dextrin	55.4	40.0	11.8
Total	100.0	100.0	100.0
Iso-caloric feeding level ¹	4.5	4.0	3.3

¹ Daily feed based on percentage of live weight. The daily allowance in calories per kilogram of live weight was approximately 165.

Normal healthy pigs were removed from the sows at the age of 3 weeks and placed in individual cages provided with wire floors, which were located in a temperature-controlled laboratory room maintained at $77^{\circ} \pm 3^{\circ}\text{F}$. Their weights ranged from 3 to 6 kg. They were assigned at random to the three diets. A limited number designated as positive controls were also fed the medium-fat diet similar to that given in table 1 except that the casein, whey, and liver were not treated to remove or inactivate the thiamine. The pigs were fed the diets at levels calculated to provide an equal caloric intake and found by earlier work to provide a full feed to the majority of animals. The figures for the feeding levels are shown in table 1. When these pigs, which had been designated to receive thiamine therapy, showed characteristic signs of depletion, the supplemental feeding of thiamine was initiated.

These signs included failure of appetite, occasional vomiting, scouring, and cessation of growth. Records of body temperature and heart-beat along with feed consumption were also kept throughout the experiment.

Thiamine therapy was based on body weight but since feed allowance was also on the same basis, the two were actually correlated throughout the therapeutic period. Most of the pigs were placed on levels of thiamine which were judged to be at or near the estimated requirements for the different diets which would give normal growth and enable the calculation of the ratio of thiamine to carbohydrate and protein as desired. The levels actually employed are shown in table 2.

TABLE 2

Results of feeding tests on young pigs showing growth response in relation to thiamine intake on different levels of dietary fat.

DIET	THIAMINE LEVEL PER KILOGRAM OF BODY WEIGHT	NUMBER OF PIGS	DEPLETION PERIOD		THIAMINE THERAPY PERIOD			
			Average time ¹	Average daily gain	Average time	Average daily gain	Feed per kg. gain of body weight	Thiamine per 100 gm. protein and carbohydrate
	<i>μg.</i>		<i>days</i>	<i>kg.</i>	<i>days</i>	<i>kg.</i>	<i>kg.</i>	<i>μg.</i>
Low-fat	0	4	25	.11
	15	1	22	.04	31	.08	2.00	55
	25	3	25	.12	39	.27	1.58	86
	40	4	24	.16	42	.42	1.47	113
	50	4	24	.11	54	.42	1.55	141
Medium-fat	0	4	28	.11
	15	1	22	.05	35	.15	1.50	54
	25	4	31	.13	48	.32	1.50	101
	35	4	26	.14	52	.46	1.27	125
	40	1	28	.14	42	.40	1.44	148
	100	1	27	.09	48	.43	1.15	341
High-fat	0	3	33	.16
	15	4	33	.12	48	.33	1.29	88
	25	4	37	.17	45	.48	1.18	135

¹ Indicates time until initiation of therapy was considered necessary. Survival times of pigs receiving no therapy averaged 39 days for low-fat, 49 days for medium-fat, and 60 days for high-fat.

They range from 15 to 100 μ g. of thiamine in the form of thiamine chloride hydrochloride daily per kilogram body weight. The test periods for the majority of animals ranged from 10 to 13 weeks for the total of depletion and therapy. The experimental work extended through four farrowing seasons from the spring of 1940 through the fall of 1941. Results on a total of forty-five pigs, including three positive controls, are available.

All animals were examined at autopsy for pathological changes. Histological studies were also made on selected tissues. Assays for thiamine content were made by the rat-growth method on samples of hams from pigs selected to represent the more critical levels of therapy.

The details of the method of assay were adapted from the procedure described by Kline, Hall and Morgan ('41). Instead of using autoclaved yeast and peanuts and sulfite-treated liver extract to supply the vitamin B factors other than thiamine, commercial dried whey and fresh hog liver were treated with a sodium sulphite solution and then dried. The rat diet consisted of 25 parts of extracted casein, 9 of lard, 20 of sugar, 20 of dextrin, 2 of agar, 3.5 of salt mixture, 0.3 of cod-liver oil, 0.7 of wheat-germ oil, 12 of sulphite-treated whey, and 7.5 (dry basis) of sulphite-treated liver.

The rats were depleted of their thiamine stores by feeding the basal diet without added supplements and then were assigned in groups to the test materials. Two and sometimes three groups were fed graded levels of thiamine chloride hydrochloride three times a week. The samples of ham tissue were weighed out and fed on the same days as the controls on thiamine. The test period lasted 4 weeks and all animals were confined in individual cages. The thiamine content of the ham samples was obtained by comparison to the reference curves based on the growth of the animals fed thiamine chloride hydrochloride.

RESULTS

The feeding of the thiamine-deficient diets resulted in essentially the same type of symptoms as those recorded in the earlier work of Van Etten Ellis and Madsen ('40). Failing appetite usually was the first sign of thiamine deprivation. There was a direct relation to level of dietary fat. On the low-fat diet the average number of days for loss of appetite to appear was 21, while on the medium and the high levels it was 25 and 31 days, respectively. Although vomiting of food was observed it did not occur in many cases. On the other hand, scouring was much more common.

Retardation of growth followed closely the failure in appetite. In table 2 are recorded the average days on test during the depletion period. The time required for unmistakable evidence of thiamine deficiency to appear generally followed within a few days the periods already given for loss of appetite and averaged 25 days for the low-fat, 28 for the medium-fat, and 33 for the high-fat diet. The average survival time for the pigs that received no thiamine was 39, 49 and 60 days, respectively, for the low-, the medium-, and the high-fat groups.

The negative control animals which were continued on the basal diet with no thiamine added were either allowed to die from the effects of thiamine deficiency or were sacrificed when in a semi-moribund condition. Inspection of the daily heart-rate and semi-weekly rectal temperature records on these animals gives further information on the course and rate of depletion of animals when fed thiamine-deficient diets of varying fat content.

Individual data for representative litter-mate negative control pigs, together with response of a deficient animal to therapy in the spring series of 1941 are shown in figures 1 and 2.

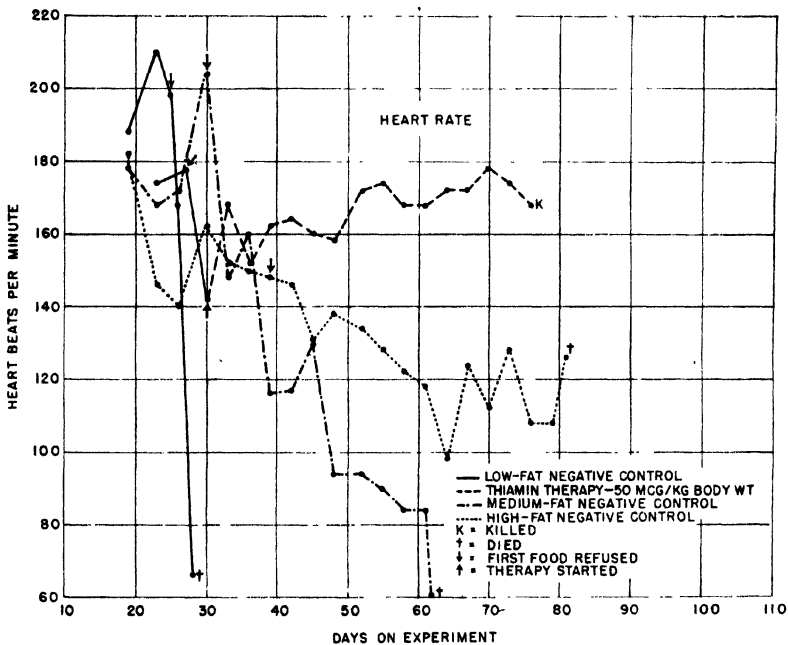


Fig. 1 The heart rates of typical individual pigs on different dietary treatments.

In figure 1 the plotted points represent the heart rate obtained by averaging values on three separate days, except in the case of the last value recorded which is the last observation before the animal died or was sacrificed.

It is obvious that slowing of the heart rate is a prominent symptom of thiamine deficiency. This became evident in the animals at about the same time that the first refusal of food was noted. Thereafter the decline in heart rate was rapid as long as no thiamine was given.

Changes in heart rate were usually accompanied by changes in rhythm and intensity of the beat. The heart of the animal on the low-fat diet failed rapidly. The animal on the medium-fat diet lived longer and had a slower rate of decrease. Similarly, the animal receiving the high-fat ration also had a gradual decrease in heart rate but the beat apparently became more variable toward the end of the survival period and did not reach as slow a rate as found for the lower levels of fat intake. In contrast the heart rate of the animal on the low-fat ration which was given 50 μ g. of thiamine per kilogram of body weight after partial depletion, rapidly returned to normal.

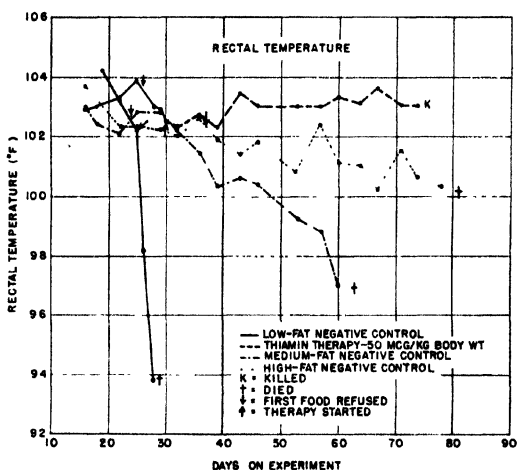


Fig. 2. The rectal temperatures of typical individual pigs on different treatments.

A similar picture is recorded in figure 2 which is a semi-weekly record of rectal temperatures taken on the same litter-mate negative control and thiamine-treated animals as shown in figure 1. The temperature of the animal on the low-fat ration dropped rapidly, the animal on the medium-fat dropped more slowly, and still more slowly for the animal on the high-fat. The temperature of the animal on the high-fat was more variable, yet higher, than in the case of the animals receiving the lower amounts of fat. The animal on the low-fat diet responded very shortly with an increase in body temperature after thiamine therapy was started.

The usual findings noted at autopsy in animals dying of thiamine deficiency were extreme emaciation, flabby heart muscle, congested liver, and occasionally free fluid in the abdominal, thoracic, and pericardial cavities.

In contrast to these negative control animals, a number of pigs fed as positive controls with the thiamine supplied through the untreated whey, liver, and casein, maintained a rate of growth approximating 0.4 of a kilogram per day over a period of 10 weeks. Furthermore, the symptoms of poor appetite, emaciation, slowing of the heart rate, and drop in temperature were absent.

Microscopical examination of a number of tissues from the negative control pigs which were usually in advanced stages of thiamine deficiency, failed to demonstrate any consistent lesions of the sciatic nerve, spinal cord, pancreas, thyroid, kidney, spleen, or adrenal glands. Various stages of myocardial degeneration were confirmed as previously reported by Van Etten, Ellis and Madsen ('40) and by Follis et al. ('43) and these changes are probably related to the slower heart rate and abnormalities of conduction previously noted by Madsen ('42) and described in this study. The heart lesions tended to be more severe in pigs fed the low- and medium-fat rations without thiamine supplement than in the high-fat negative controls. Small, apparently healed areas consisting of atrophy or complete disappearance of cardiac muscle fibers often accompanied by an infiltration of mononuclear cells were seen in the hearts from some of the pigs that had been partially depleted of thiamine and then fed adequate levels of this vitamin. This suggests that injury to the heart may occur along with the other early criteria of deficiency such as inappetence and slowing of the growth rate or less in weight. Varying degrees of atrophy of the gastric glands in the pyloric region were noted in most of the thiamine-deficient pigs. The most severe case occurred in a negative control pig on the high-fat ration, that died after 82 days on the deficient ration.

THIAMINE THERAPY

In most cases, the animals depleted on the thiamine-low diets responded immediately to the feeding of thiamine. The appetites returned to normal, vomiting usually ceased, the body temperatures returned to normal, and resumption in growth was noted within a week. There was a striking and characteristic difference in the performance of the animals fed the same amount of thiamine on the body weight basis but on different levels of dietary fat. These differences are shown in table 2. For example, 25 μ g. of thiamine were given to pigs on all three diets. The average daily gains increased from 0.27 kg. on the low-fat diet to 0.48 kg. on the high-fat. To produce approximately the equal of the gain noted on the high-fat diet required the feeding of at least 40 μ g. of thiamine on the low-fat and 35 on the medium-fat diet. At these levels the thiamine consumption per 100 gm. of protein

and carbohydrate was not greatly different since it fell within the range of 125 to 141 μg . These figures are somewhat higher than the range of 106 to 120 μg . previously suggested and probably allow a somewhat greater margin above the bare requirement for normal growth. It is noteworthy that the daily gains made by the pigs on the higher thiamine levels were approximately 1 pound, a rate much above the usual average for pigs of 6 to 13 weeks in age. The data for economy of feed utilization shown in table 2 also reveal some unusual figures which are probably attributable to the confinement of the pigs in a room maintained at a uniform temperature. At the time of slaughter,

TABLE 3

Electrocardiograph readings on pigs with and without thiamine present in their ration.

PIG NUMBER AND DIET TREATMENT	HEART RATE	P-R INTERVAL	Q-T INTERVAL	Q-T INTERVAL CORRECTED FOR CYCLE LENGTH=X	ALGEBRAIC SUM OF QRS IN LEAD I	ALGEBRAIC SUM OF QRS IN LEAD II	ALGEBRAIC SUM OF QRS IN LEAD III
	<i>beats per min.</i>	<i>sec.</i>	<i>sec.</i>	<i>K.</i>	<i>millivolts</i>	<i>millivolts</i>	<i>millivolts</i>
A-low fat, no thiamine	68	0.10	0.34	0.37	+ .15	+ .02	0.0
B-medium fat, no thiamine	85	0.14	0.35	0.40	— .29	+ .13	+ 0.64
C-medium fat, 25 μg . thiamine	125	0.10	0.27	0.39	+ .14	+ .63	+ .60
D-positive control thiamine present . . .	138	0.10	0.21	0.34	+ .38	+ 1.10	+ .47

the pigs which had received levels of thiamine adequate for growth were fully as fat if not fatter than the average pig in the herd from which these were obtained.

Through the courtesy of Dr. H. O. Calvery and Dr. Bert J. Vos, Jr., of the Food and Drug Administration, electrocardiograms were secured on four pigs fed during the spring of 1940. The data are given in table 3. Two of the pigs (designated as A and B) had been on low-thiamine diets for 50 days, had been losing weight for approximately 3 weeks and generally showed the usual advanced symptoms of thiamine deficiency. Pig C, at the time the electrocardiograms were made, had been receiving 25 μg . of thiamine per kilogram of body weight for approximately 2 weeks following a 4-week depletion period, and had fully recovered his appetite and general state of well-being. The fourth pig had been fed for about 50 days on the positive-control diet with adequate amounts of thiamine present. All four animals had been handled daily and were, therefore, good subjects for study. Very little

difficulty was encountered in making the electrocardiograms on the two thiamine-deficient pigs. Pig D, the positive control, was the most active and the most difficult to restrain while the readings were being made.

The heart rate as shown in table 3 was unmistakably slower in the two pigs which had been deprived of thiamine than in the two with the vitamin present in their diet. Pig B had a right axis deviation as shown by the negative value of the algebraic sum of QRS in lead 1. This finding is frequently associated with hypertrophy of the right ventricle.

TABLE 4

Influence of level of thiamine fed to pigs on the content of this substance in ham muscle.

LEVEL OF THIAMINE FEEDING (MICROGRAMS PER KILOGRAM OF LIVE WEIGHT OF PIG)	MICROGRAMS OF THIAMINE PER 100 GRAMS OF HAM MUSCLE IN PIGS FED DIET DESIGNATED		
	Low-fat	Medium-fat	High-fat
0	..	Trace	Trace
15	4 ¹
25	19	24 ¹	35 ¹
35	..	33 ²	..
40	17	60	..
50	39 ¹
100	..	168	..

¹ Represents average of two pigs.

² Represents average of three pigs; all other data are for one pig each.

The K values, as indices of the speed of ventricular contraction, were slightly higher in pigs A and B than in pig D. The high value for pig C, which had been receiving thiamine for approximately 2 weeks, however, suggests that the differences may be due to random variability unless the therapy period of pig C had been insufficient to allow for correction of the abnormality.

These data are believed to be fully in line with other evidences of heart damage, not only in pigs but in other animals.

The assays for thiamine content of the lean tissues from the hams of a number of the experimental pigs are shown in table 4. Comparable assays on two herd pigs that had been fed an ordinary diet of corn and supplements and slaughtered at the same ages as the experimental pigs, gave values of 785 and 795 µg. of thiamine per 100 gm. of fresh tissue. These values are in turn somewhat lower than found in older pigs weighing 225 pounds, where the values range from 690 to 1520. Compared to these values, the experimental pigs did not store appreciable amounts of thiamine although the figures in table 4 show a gradual but consistent increase in thiamine content of meat tissue with increase in dietary fat and thiamine intake. It is evident that the thia-

mine requirements for maintenance and growth are met before appreciable storage takes place and furthermore that the extent of storage is proportionate to the level of thiamine. The low figures for storage of thiamine are in harmony with those of Hughes ('41). That even commercial pork may vary in thiamine content through wide limits has been shown by Waisman and Elvehjem ('41) who have reported values on pork hams ranging from 910 to 1900 $\mu\text{g.}$ per 100 gm. of fresh tissue.

SUMMARY

The thiamine requirements of young pigs have been studied on three diets containing approximately 2, 11, and 28% of fat. As indicated by failure in appetite and cessation of growth, the animals on the low level of fat showed evidence of thiamine depletion on the average in 25 days, those on the medium level in 28 days, and on the high level in 33 days. Lack of thiamine resulted in marked weakening of the heart, decrease in body temperature, emaciation, and other changes.

When thiamine was fed to pigs depleted of their stores of this substance, the response in appetite, growth, and general health was usually prompt and striking. Intermediate levels of thiamine produced the greatest response in the pigs fed the high-fat diet, followed in order by those on the intermediate and the low-fat. It was found that the level of thiamine required to produce a maximum rate of growth and otherwise maintain the pigs in good health fell within the range of 125 to 141 $\mu\text{g.}$ per 100 gm. of carbohydrate and protein. These levels of thiamine, however, were insufficient to promote the storage of normal amounts in the meat tissue such as is found in commercial pork cuts.

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INTERRELATION OF METHIONINE, CHOLINE, BETAINES AND ARSENOCHOLINE IN THE CHICK

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In a preceding report (Grau and Almquist, '43) we have shown that the choline-deficient chick can utilize homocystine in lieu of cystine, but not in lieu of methionine. Results of additional studies, which include the effect of methionine, betaine and arsenocholine as substitutes for choline, may now be presented. The general procedure and the basal diet have been described in detail (Grau and Almquist, '43). Briefly, choline-depleted chicks were fed a synthetic-type diet in which the protein source was an isolated soybean protein of known methionine and cystine contents. The three or four chicks used in each group were carefully selected with respect to weight, rate of gain in weight, and degree of choline depletion. The experiments were carried on for 1 week, during which time the chicks were weighed daily.

Some of the data now reported were obtained simultaneously with, and all are directly comparable to, those in the preceding report. For convenience in making comparisons, the diets have been numbered in a continuous series with those of the first paper, from which report pertinent data have been summarized in table 1. Percentage daily rate of gain will be referred to simply as gain.

A preliminary communication (Almquist and Grau, '43) has been made on the growth-promoting effect of betaine (glycine betaine). It was reported that under certain conditions betaine may promote growth as effectively as choline or methionine. Earlier reports of a growth-promoting effect of betaine in chick diets have been made (Jukes, '40; Jukes and Welch, '42; McGinnis, Norris and Heuser, '42).

RESULTS

The extended studies with betaine (table 2) demonstrate a growth-promoting action which becomes conspicuous under conditions of choline and methionine deficiency. Betaine alone caused no appreciable increase in gain (compare diets 1 and 24). Insofar as the added sulfur

amino acids consisted of cystine or homocystine or both, the addition of betaine allowed gains of approximately 4.0% per day. The increases in gain due to betaine addition were approximately 2.0 throughout (compare diets 24 and 25, 5 and 26, 6 and 28, 10 and 29, 11 and 30). In the presence of methionine, however, the addition of betaine led to very little further increase in rate above 4.0 (compare diets 12 and 32). All of these diets, evidently, lacked some growth-promoting substance, the effect of which was not appreciably provided even by excesses of betaine or methionine, but which was provided by the diets containing choline (i. e., diets 19, 21, 22, 23 and 41).

TABLE 1

Summary of pertinent data from the report of Grau and Almquist ('43).

DIET. NUMBER	SUPPLEMENTS ADDED TO THE BASAL DIET ¹				TOTAL MILLIMOLS SULFUR AMINO ACIDS ADDED PER 100 GM. DIET	AVERAGE % GAIN PER DAY
	l-Cystine	dl-Homo- cystine	dl-Meth- ionine	Choline chloride		
1	1.2
2	0.20	1.67	1.8
3	0.40	3.34	1.9
5	...	0.25	1.72	1.8
6	...	0.45	3.36	1.5
7	0.25	...	1.68	3.7
8	0.50	...	3.36	4.2
9	1.00	...	6.72	4.3
10	0.20	0.23	3.39	2.0
11	0.20	0.45	5.03	1.6
12	0.20	...	0.50	...	5.03	4.4
19	0.20	0.45	...	0.20	5.03	6.4
21	0.20	...	0.50	0.05	5.03	6.0 *
22	0.20	...	0.50	0.20	5.03	6.0
23	0.20	...	0.70	0.20	6.37	6.3

¹ Expressed in per cent added to diet.

Arsenocholine additions, as in the case of betaine and methionine additions, without choline, led to similar gains, approximately 4.0, by the chicks on diets containing added cystine and homocystine (diets 34, 35, 36 and 37). An important difference from the betaine results was observed in the case of diets 38 and 42, where the substitution of arsenocholine for betaine in the presence of added methionine led to definitely better growth. More striking, however, were the results with the combinations of betaine and arsenocholine, which yielded optimal gains (compare diets 37 and 39; also diets 32 and 44).

TABLE 2
The relation of methionine, betaine and arsenocholine to the choline requirement of the chick.

DIET NUMBER	l-Cystine	dl-Homocystine	di-Methionine	Betaine hydrochloride	Arsenocholine chloride	Choline chloride	TOTAL MILLIMOLES SULFUR AMINO ACIDS ADDED PER 100 GM. DIET	NUMBER OF GROUPS	AVERAGE % GAIN PER DAY
24	0.22	3	1.4
25	0.20	0.22	1.67	3	3.4
26	...	0.23	...	0.22	1.72	1	4.1
27	...	0.23	...	0.30	1.72	1	4.0
28	...	0.45	...	0.22	3.36	1	3.6
29	0.20	0.23	...	0.30	3.39	1	4.1
30	0.20	0.45	...	0.22	5.03	2	4.1
31	0.20	0.45	...	0.50	5.03	1	4.0
32	0.20	...	0.50	0.22	5.03	2	4.6
33	0.20	...	0.70	0.22	6.37	1	4.8
34	0.20	0.29	...	1.67	1	3.8
35	0.40	0.39	...	3.34	1	3.8
36	0.20	0.23	0.30	...	3.39	1	3.8
37	0.20	0.45	0.29	...	5.03	1	4.2
38	0.20	...	0.50	...	0.29	...	5.03	1	5.7
39	0.20	0.45	...	0.22	0.29	...	5.03	1	6.6
40	0.20	...	0.50	0.22	...	0.02	5.03	1	5.3
41	0.20	...	0.50	0.22	...	0.05	5.03	1	6.0
42	0.20	...	0.75	...	0.29	...	6.42	1	5.8
43	0.20	...	0.50	0.22	0.07	...	5.03	1	5.3
44	0.20	...	0.50	0.22	0.14	...	5.03	1	6.3

¹ Expressed in percent added to diet.

DISCUSSION

The growth-effect of betaine is not readily accounted for on a basis of facilitating choline synthesis in the chick. This is indicated by the fact that betaine is not appreciably effective in the prevention of choline-deficiency perosis (Jukes, '40; Jukes and Welch, '42).¹ Other evidence showing a lack of ready choline synthesis in the chick, even in the presence of an excess of methionine or of methionine plus ethanolamine, is available (Jukes, '41; Record and Bethke, 42).²

It is noteworthy that the increases in gain upon addition of betaine were but slightly better with homocystine than with cystine (compare diets 2 and 25, increase in gain 1.6%/day; with diets 5 and 26, increase 2.3; diets 6 and 28, increase 2.1; diets 11 and 30, increase 2.5). An excess of betaine (diet 31) or of homocystine (diets 30 and 31), in relation to gain observed, did not seem to increase the gain further. That incomplete methylation of homocystine is not the full explanation for the lack of optimal gain is shown by the fact that replacement of the homocystine by methionine (diet 32) permitted only a slightly better gain, which was still far below the optimum.

That methionine exerts an effect similar to that of betaine is indicated by the results with diets 7, 8 and 9, as compared with 2. In the case of diets 7, 8 and 9, practically all sulfur amino acids were in the form of methionine, some of which must have been converted *in vivo* to cystine. These diets could hardly have been expected to be so markedly superior to diet 2, which had 0.20% cystine added to it, and also contained 0.30% methionine present in the basal diet. Lack of certain choline functions is indicated here also, for the gains with any level of methionine did not appreciably exceed 4.0% per day. Furthermore, betaine and methionine fed in combination did not provide the missing effects of choline (diets 32 and 33).

Since the conception that betaine may serve as a precursor of choline in the chick appears either untenable or inadequate, on the basis of

¹ McGinnis, Norris and Heuser ('42), using a diet differing greatly from that of Jukes, reported a positive antiperotic action of betaine and indications that betaine and ethanolamine fed jointly were somewhat more effective than betaine alone. In view of this apparent controversy we repeated the studies of Jukes with choline and betaine and completely confirmed his original findings. The combination of betaine and ethanolamine was also found to be ineffective in preventing perosis. Professor Norris has indicated in private correspondence that antiperotic effects with betaine were not obtained with still another type of diet and that an explanation of the results first reported is being sought.

² We may also call attention to further data confirming the above reports on the lack of effective antiperotic action by betaine, methionine or their combinations with ethanolamine, provided in the Nutrition News Letter no. 11 of the Archer-Daniels-Midland Co., Minneapolis, Minnesota, under the date of May 28, 1942.

present knowledge, it is difficult to explain the effects of betaine except by assuming that it is physiologically important to the chick. It is possible that betaine may even be formed in the chick from choline or methionine. Whatever may be the explanation, all three of these substances are capable of exerting a similar growth-promoting effect which we shall designate as B (betaine effect). This effect B is undoubtedly the cause for earlier results indicating that betaine gave growth effects which were either small or of doubtful significance (Jukes, '40; Jukes and Welch, '42; McGinnis, Norris and Heuser, '42).

In contrast to betaine, arsenocholine has been reported to be strongly antiperotic and growth-promoting in the fowl (Jukes, '40; Jukes and Welch, '42). It is unable to promote appreciable methylation of homocystine in the chick (Almquist and Jukes, '43) or in the rat (Welch, '41; Moyer and du Vigneaud, '42). Arsenocholine enters into the synthesis of phospholipids as a structural component replacing choline (Welch and Landau, '42), hence it may be particularly useful to the choline-deficient chick, in which choline synthesis appears, at best, to be a very inadequate process.

In the case of diets containing arsenocholine without betaine or added methionine, distinct but limited increases in gain (approximately 2.0% per day) were observed (compare diets 2 and 34, 3 and 35, 10 and 36, 11 and 37). Arsenocholine in the presence of added methionine caused an appreciably greater increase in gain (diet 38) than did betaine in a similar diet (32) (compare also diets 42 and 33). Most striking of all was the optimal growth obtained with the combination of betaine and arsenocholine (diets 39 and 44). It is evident that arsenocholine imparts certain additional growth-promoting influences which we shall designate as A (arsenocholine effect).

Our present concept is that arsenocholine provides effect A but very little effect B; hence growth with arsenocholine is limited by the lack of effect B. The converse of this seems true with betaine and methionine. In the presence of betaine or methionine or both, the effect of arsenocholine is additive, and the gains are similar to those obtained with choline, which provides both effects A and B. Each effect is undoubtedly multiple in nature.

Data showing a normal retention of bound choline in the liver and muscle tissues of severely perotic chicks reared on choline-deficient diets have been kindly made available by Dr. Jukes.³ His comment is of interest: "... choline is a 'building stone' and if it is deficient less tissue is formed." It seems highly probable that bound choline in tissues con-

³ Private correspondence.

stitutes the primary demand on choline; hence substances which will spare or simulate other functions of choline in a choline-depleted animal produce pronounced effects. Such sparing action may be included in both effects A and B. One must also recognize the possibility that tissue-bound choline may be able to promote methylation processes and growth may then ensue if further functions of choline are provided by suitable substitutes.

In the presence of an ample source of effect B (diets 21 and 41), 0.05% of choline chloride sufficed for approximately optimal gain, which suggests that this amount of choline was sufficient to provide effect A. It seems probable that the level of arsenocholine used in these studies was also ample to provide effect A, except in the case of diet 43. On a molar basis, arsenocholine appears to be approximately one half as active as choline in providing effect A.

The minimum amount of methionine required in the diet of the chick for optimal gain is approximately 0.55%, as pointed out in the preceding report. In the case of diets such as 19, in which optimal gain was obtained with homocystine and choline additions, approximately half of the homocystine must have been methylated to provide an adequate level of total methionine (0.30 in the basal diet plus 0.25 by methylation of homocystine). The remainder of the homocystine converted to cystine would then complete the sulfur amino acid requirement of the chick. In the case of diet 39, which contained betaine and arsenocholine in place of choline, it is apparent that a similar conversion of the homocystine to methionine and cystine must have taken place. This implies that appreciable methylating power may be ascribed to betaine (or to tissue-bound choline) in the choline-depleted chick.

When the criterion for transmethylation is growth of animals on a choline-deficient, homocystine-supplemented diet, it appears possible that the methylating power of compounds may be underestimated by virtue of a deficiency of certain of the growth-promoting influences of choline. In such studies the inclusion in the diet of some relatively non-methylating substance such as arsenocholine (to provide effect A) would seem advisable.

The results of these studies show that the practical dietary choline requirements of animals may be modified by the adequacy or deficiency of betaine and methionine. Betaine is of widespread occurrence in feedstuffs. The choline-like activities of practical feedstuffs are, in all probability, the resultant of the combined effects of choline, betaine and methionine. The present work furnishes no clear information on a methionine-sparing action of choline and betaine. This will require

further study. However, a choline-sparing action on the part of methionine and betaine is distinctly indicated.

SUMMARY

1. Betaine (glycine betaine) and methionine are capable of assuming a certain portion of the functions of choline in the chick.

2. Arsenocholine is capable of assuming the remainder of the choline functions.

3. The effects of betaine (or methionine) and arsenocholine are additive, and, together, are practically a complete substitute for choline under the conditions of these experiments.

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THE MINIMUM ASCORBIC ACID NEED OF ADULTS¹

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FIVE FIGURES

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In spite of the extensive literature on human nutrition there is still need for more data on minimal safe levels of intake of the essential food factors. Early in 1942 we conceived the idea of using male prisoners as subjects for a study of some of the basic nutritional requirements of healthy adults. The handicap of a 55-mile distance separating the Wisconsin State Prison and our laboratory was largely compensated for by the cordial assistance given us by the warden and medical staff of the prison hospital. Subjects were selected from volunteers after the project had been explained to the entire inmate population of more than 1100 over the prison radio. The group was more than ordinarily homogeneous in that the men were on a controlled and regular regime. They were regularly available to our convenience for examination and tests; their cooperation was excellent. Information concerning diet, habits, history and physical condition was at hand.

A preliminary study was made on a group of fifty-six men whose records showed no major illnesses for at least a year. Nearly all were of military age and had a fair to good I.Q. rating. All had been in prison for at least 1 year with sentences not to be completed for a year or more. Before the preliminary survey was completed it became obvious that most of the examination records indicated the presence of oral disease. We found almost universally extremely low plasma ascorbic acid values. Therefore, in May, 1942 a more detailed investigation of the Vitamin C factor was begun. This paper reports the findings in our ascorbic acid studies.

PROCEDURE

Intensive study, which included 778 determinations of plasma ascorbic acid, was made of seventy-one subjects. We were furnished menu sheets

¹This study has been supported by special grants from the Wisconsin Alumni Research Foundation and from the Nutrition Foundation to one of us. (E.L.S.)

²Major, M.C., U.S.A., 44th General Hospital.

listing every item of food for each meal served to all inmates from March, 1942 to July, 1943. Records were kept of all fruits eaten in addition to regular diet.

At the first examination each subject's gums were inspected and the observations recorded. Tourniquet tests were done but were obviously unsatisfactory, and consequently were not repeated. Blood samples were obtained at least 3 hours after breakfast. The amounts of ascorbic acid in this meal were so small that the blood values would not have been materially altered in any case. Mindlin and Butler's ('38) photo-colorimetric method was used without the KCN as an anti-oxidant. Scrupulous precautions were taken to guard against hemolysis when the blood was drawn, since this is known to cause loss of ascorbic acid by oxidation, catalyzed by the red cell contents. Blood specimens were iced immediately and brought to the laboratory on ice within 3 hours after withdrawal.

Vitamin C in drawn blood has been found in our laboratory and elsewhere to be stable within practical limits for many hours providing, as was our practice: (1) that the plasma is not separated from the cells when the sample must stand; (2) that there is no hemolysis; and especially (3) that, in addition, the blood is kept cold when it cannot be sterile. It has also been known for a long time that the vitamin is stabilized in the metaphosphoric acid filtrate.

To test the safety of carrying whole blood on ice for delayed analysis, twelve samples were drawn in such quantity that part could be taken to our laboratory in the usual way, and another portion centrifuged at the prison and the protein-free plasma filtrate made promptly for analysis as a duplicate after return from the prison. The results in these duplicate analyses agreed within the margin of error of the method. Differences in all cases were less than 0.1 mg. % which is the maximum variation between aliquots from the same blood in our use of the Mindlin-Butler method. Two showed no difference, and eight were less than 0.04 mg. % apart, with some figures higher and others lower in the filtrates promptly made. Keeping qualities by either way of handling were therefore judged to be satisfactory, so that we pursued the far simpler method of delaying the whole analysis until our return to the laboratory.

A further partial check on the effect of delayed analysis as such was made by taking samples from three of the authors while at the prison, and comparing these results with those of promptly analyzed samples obtained on the same individuals at the same time on the following day while they were in the laboratory. The repeated analyses

agreed within 0.04, 0.09, and 0.01 mg. %. Although the promptly analyzed sample in each case was the higher, the difference was certainly well within the expected error of the method in routine use.

Before supplements of ascorbic acid were given, each man had two or more control plasma vitamin C values recorded. Blood specimens were obtained usually once a week during the first 8 months of the study, thereafter less often. Four or more untreated subjects were included with the supplemented ones to serve as controls on the possible effects of season or changes in the institutional diet (fig. 1). An inspection

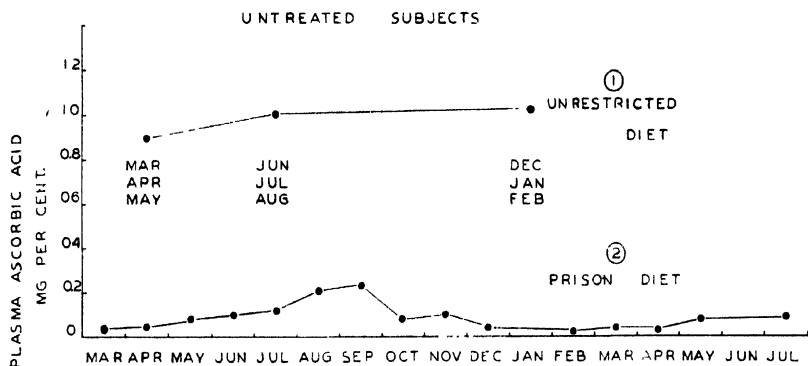


Fig. 1 Comparison of (1) the seasonal (spring, summer, winter) averages of single plasma ascorbic acid values on a miscellaneous group of thirty-one medical and home economics students and (2) the monthly averages of untreated subjects on prison diet, with or without occasional fruit intake. Subjects were excluded who reported daily use of vitamin preparations or fruit equivalent to one orange daily.

of the gums was made each time. Note was made of extra fruit eaten or of any change in health status. Of the seventy-one men, on first examination 77% had a plasma ascorbic acid level below 0.2 mg. %, 20% had between 0.2 and 0.6 mg. %, while only two (3%) had over 0.6 mg. %.

RESULTS

In May, 1942, twelve men were started on 25 mg. of synthetic ascorbic acid daily. The prison hospital staff supervised the daily distribution of tablets and made certain that each man was actually taking his dose. Control plasma values for this group were all below 0.2 mg. %. After 5 weeks the rise was insignificant. Accordingly, the daily dose was increased to 50 mg., which in 2 weeks' time definitely elevated the plasma level (fig. 2).

Other groups were started meanwhile on daily supplements of 50 mg. and 75 mg. Much individual variation in response to the same dose was noted. There were forty-five men who were available for the 6 to 14 months' period of supplementation necessary to establish and maintain a satisfactory plasma ascorbic acid level for several weeks

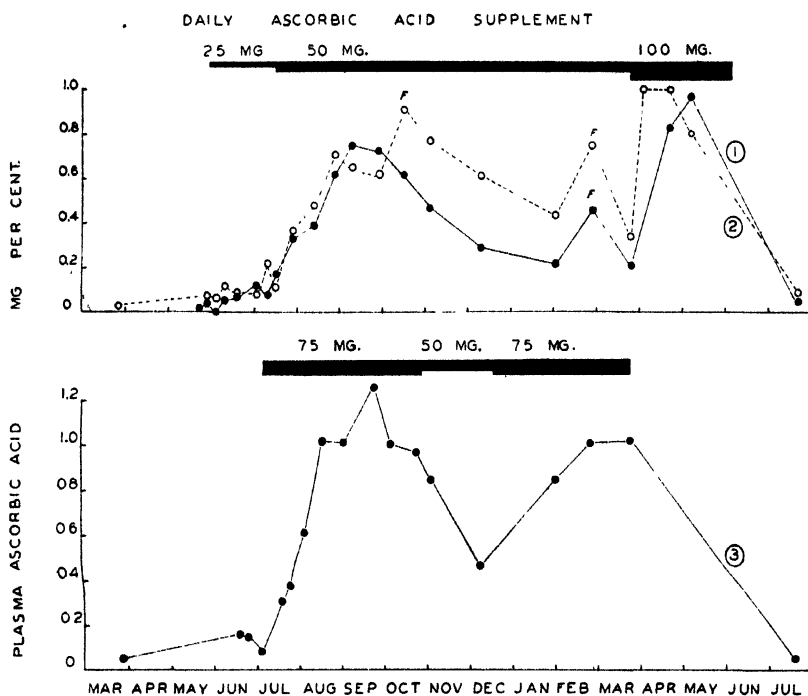


Fig. 2 Typical individual curves of blood response to different amounts of supplement.

Curve (1), subject no. 50, age 22, prison inmate 3 years.

Curve (2), subject no. 8, age 32, prison inmate 11 years.

Curve (3), subject no. 9, age 25, prison inmate 2 years.

(F) indicates recent intake of citrus fruit.

and to show satisfactory improvement of the gum conditions. Unexpected causes such as parole or removal to one of the prison farms interrupted the study of a few others. The rest studied were untreated control subjects. Among the forty-five cases, there were three subjects whose plasma response was unsatisfactory until the daily supplement was increased to 100 mg. (fig. 3). Observations of the gums of these men while taking 50 and 75 mg. revealed a lack of the usual improvement noted in the others receiving the same amount of ascorbic acid. Further

investigation proved all three to have a history of chronic nose and throat infections, and in two of the men severe infection was also present in the gums. Among the other few who complained of frequent colds, none exhibited definite signs of such chronicity. There were other subjects whose gums appeared as severely affected as (or more so than) those of the three refractory subjects, but as we did not attempt any bacteriological study of the infecting organisms, it is difficult to say how much bearing the type of organism may have had on the question.

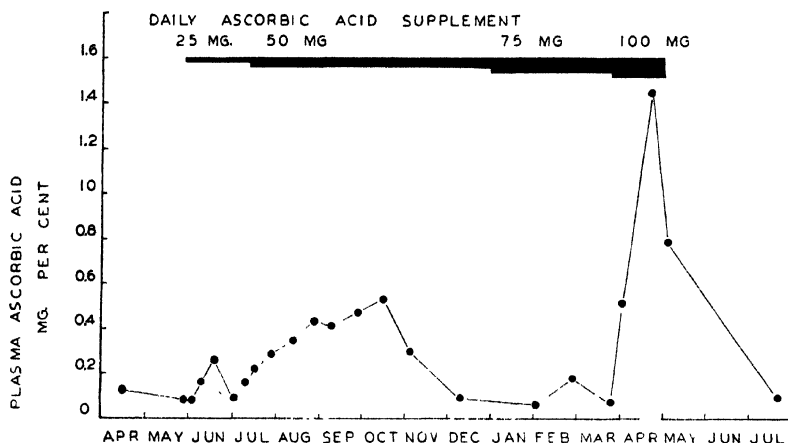


Fig. 3 Unsatisfactory blood response to supplements of 75 mg. or less. Subject no. 26, age 34, prison inmate 9 years, with a history and physical signs of chronic nose and throat infection; also advanced oral disease ("pyorrhea" cause of extraction of six lower front teeth).

Throughout August and September plasma ascorbic acid of the majority of subjects on 50 mg. supplements was maintained between 0.7 and 1.0 mg. % (fig. 2). This was the season in which a few raw vegetables such as onions and cabbages were on the menu. There may also have been an increase in the vitamin C content of the potatoes at the time, and in addition, more fruit was reported eaten as extras. The incidence of infections such as colds, was lower. Through the other months 50 mg. of supplement was inadequate (fig. 2). A slight seasonal rise for August and September may be seen on the curve (fig. 1) of the untreated controls.

A few subjects were carried through the winter on 50, 75, or 100 mg. of ascorbic acid daily. Upon withdrawal of supplements, a group of eighteen were given placebos. Typical results are illustrated (fig. 4).

Then, early in May, 1943, all supplements and placebos were withdrawn. The men were notified of the end of restrictions on extra fruit and vitamin pills. A last check was made late in July to determine the status of as many as possible. Thirty-four men were seen at this time. Plasma ascorbic acid levels of 28 (82%) were again below 0.2 mg. %; none were higher than 0.5 mg. %.

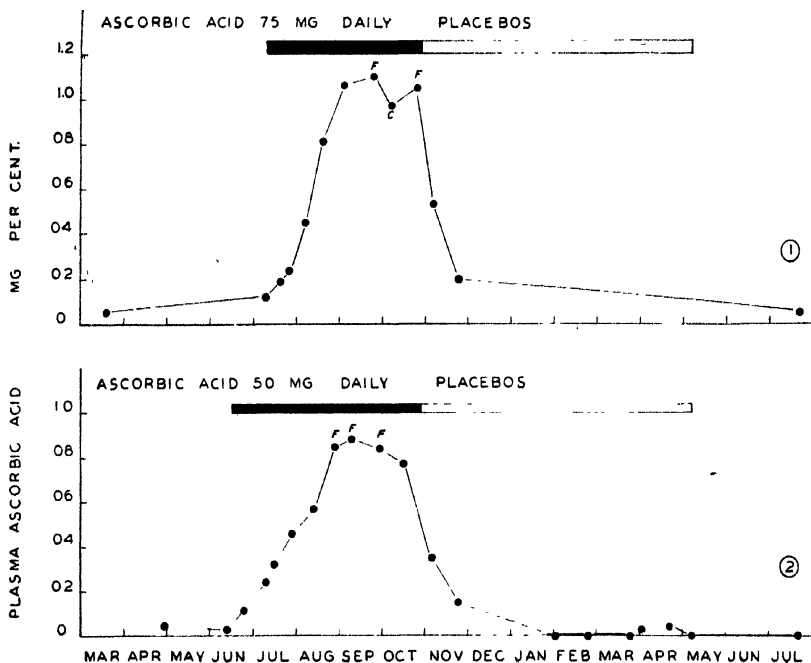


Fig. 4 Effect on plasma ascorbic acid of placebo substitution for supplements.

Curve (1), subject no. 3, age 27, prison inmate 6 years.

Curve (2), subject no. 41, age 31, prison inmate 9 years.

(F) indicates recent intake of citrus fruit.

(C) indicates recent nose and throat infection.

DISEASED GUMS

The most striking observation of the entire study was the relationship between the plasma ascorbic acid level and the health of the gums. Figure 5 illustrates a typical case in which are shown blood levels and brief notes of gum conditions made in corresponding months. Notes on the gums were made at the time of blood sampling and recorded before plasma values were known. One of us (E.D.K.) made all of the regularly recorded gum examinations. Three independent observations were

made by another (E.L.S.), two by E.S.G., and on another occasion we are pleased to acknowledge the opinions of Dr. T. A. Hardgrove, who has given considerable attention to the possible connection between dietary deficiency and gum conditions. Substantial agreement was noted by all four observers. We recognize, of course, that there are cases of gum disease which cannot be correlated with a history of low fruit intake or low plasma ascorbic acid values, and which do not respond to treatment with large doses of ascorbic acid.

The preliminary survey inspection revealed one subject whose gums appeared normal and free from disease. His plasma ascorbic acid reading was 0.95, the highest figure encountered in any untreated

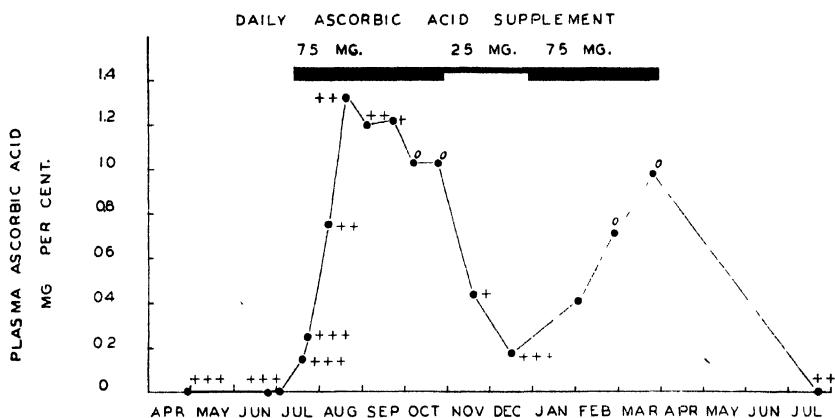


Fig. 5 Subject no. 39, age 25, prison inmate 2 years. Plasma ascorbic acid curve with gum changes indicated as follows:

Moderate to severe oral disease +++; slight to moderate ++; slight +; no disease 0.
4-30-42 — Marginal infection and recession — lower anterior, pallor with reddened and a few cyanotic areas, some edema, bleeding.

6-24, 7-1, 7-17 — No change.

7-22 — Color slightly improved.

8-5 — Color improved, no cyanosis, less edema, infection disappearing.

8-19 — Gums bleed only when "brushed violently"; general appearance shows marked improvement.

9-2 — Progressive improvement, no bleeding, margins not entirely healed.

10-23 — Gums appear normal.

11-19 — Lowers show abnormal redness, marginal pallor.

12-16 — Increasing gingival irritation, slight infection at margin of left lower bicuspid.

2-25-43 — Gums normal.

3-24 — No change.

7-22 — No bleeding, some edema, poor color, marginal pallor, suggestion of recession (recurrent).

(Reports taking twenty-four vitamin pills since May).

prison subject. On his history sheet we found his report of regular daily consumption of one or two oranges for over 2 years.

Oral hygiene had been grossly neglected in a number of cases but at least half of the mouths examined had fair to excellent care. Bleeding of the gums on brushing was a common report. In fact small hemorrhages were visible on gums that had obviously been recently cleaned. Nine of the men were edentulous; six of this number were between 30 and 40 years of age. Many others had lost more than a third of their teeth. The reason for extraction was practically always recorded as "on account of pyorrhea". This complaint was so unusually frequent for men in their late 20's and early 30's that it appeared pertinent to inspect the details of the diet regularly served.

There was very little variation in the diet from week to week. Winter diet differed little from the summer diet. No fresh fruit or fruit juices and only small amounts of raw vegetables were listed. A typical week's diet is illustrated in table 1.

Furthermore, the preparation by steam cookery and the delay between preparation and consumption, which is unavoidable in the service of a large institution, make it improbable that more than a few milligrams of vitamin C are ingested per man through a day's food ration. Lack of teeth, of course, interfered with food intake, so that a "vicious circle" was established that interfered further with good nutrition in many of the inmates.

Gum inspection after a month's time on 25-mg. dose showed definite improvement of the oral tissues, even though little rise had occurred in the plasma vitamin C curve. The trend toward a normal pink color could be seen. Edema had decidedly subsided, particularly in mouths originally not too severely affected. With doses increased to 50 or 75 mg., as the rise in the plasma ascorbic acid became more pronounced, improvement in the appearance of the gums was more marked. Severe bleeding of the gums disappeared after 2 or 3 months if the blood ascorbic acid level was maintained in the neighborhood of 1.0 mg. %. In about the same period there was noted a tendency for pus pockets to clear, and if the normal blood level was maintained still longer some cases healed and new tissue appeared. Rise in the plasma values is reflected later in the improved appearance of affected gum tissues. The lag in signs of improvement was most pronounced in the individuals whose plasma values were most rapidly increased at the start by the larger doses. A similar lag in regression after the drop in plasma values occurred on withdrawal of supplements. Effect of withdrawal of the ascorbic acid supplement was noted in the plasma curves within

TABLE 1

Illustrated menus for the week ending June 5, 1943.

Sunday	Kix cereal Milk and sugar Stewed prunes Sweet rolls (2) Bread — butter Coffee	Baked swiss steak Brown gravy Mashed potatoes Creamed corn Rhubarb pie Bread — coffee	Sliced cold meat Fried potatoes Spice cake Raisin sauce Bread — syrup Coffee
Monday	Wheat cakes Syrup Bread Butter Coffee	Baked pork chop Brown gravy Oven brown potatoes String beans Apple cake Bread Coffee	Potato salad Sliced liver sausage Cup cakes Prune sauce Bread Syrup Coffee
Tuesday	Cream of wheat Milk and sugar Scrambled eggs Bread Butter Coffee	Baked Polish sausage Sauer kraut Steamed potatoes Rice pudding Bread Milk	Chili con carne Croutons Stewed apples Muffins Bread — syrup Coffee
Wednesday	Baked meat balls Fried potatoes Doughnuts Bread Syrup Coffee	Braised beef stew Mashed potatoes Beet greens Lemon pudding Bread Coffee	Wheat cakes Cold meats Stewed prunes Bread — butter Syrup Coffee
Thursday	Boiled oatmeal Milk and sugar Raisin sauce Filled rolls (2) Bread Butter Coffee	Breaded pork steak Lyonnais potatoes Cold tomatoes Cream pie Bread Coffee	French toast Cold meat Fried potatoes Apple sauce Bread Syrup Coffee
Friday	Wheat cakes Syrup Bread Butter Coffee	Baked halibut Mashed potatoes Creamed peas Butterscotch pudding Bread Coffee	Sliced American cheese Cookies Rhubarb sauce Bread Syrup Coffee
Saturday	Baked franks Hashed brown potatoes Hard rolls Bread Butter Coffee	Hamburger steak Oven brown potatoes Stewed carrots Orange pudding Bread Coffee	Baked pork and beans Doughnuts Cottage cheese Raisin sauce Bread — syrup Coffee

a week in our subjects. Plasma ascorbic acid dropped sharply below the "borderline" 0.8 level even if it had been carried for some time at a higher level. Four to 5 weeks after placebos were substituted for the supplements, plasma ascorbic acid was 0.2 mg. % or less for all subjects. The gums that had been normal in appearance again exhibited a slight puffiness and abnormal redness with occasional cyanosis.

Perhaps we did not give large enough doses to observe the striking results mentioned by Hanke ('30). But the types of lesions he describes were seen over and over. The most rapid recoveries were in the gums he calls the "soft and sometimes hypertrophied type" which are "very common" and show the smallest initial degree of tissue change. His "spongy gum" type represents a greater degree of involvement and is the gum that bleeds. This symptom when present did not disappear as rapidly as did edema and acute redness on treatment with supplements.

Comparing the notes and figures for July, 1943 with the original findings of May, 1942, we found that although the plasma ascorbic acid had dropped back to its original low level, the deterioration of the oral tissue, while pronounced, was by no means as severe in general, as the conditions originally observed. One suggestion we made had resulted in the serving of a slightly improved diet in the institution, which apparently somewhat more than compensated for the fact that less fruit is bought at present due to high prices. Beginning in the winter of 1942-43 canned tomatoes, served without reheating, have been offered in three or four meals a week. It was not formerly a popular food but is apparently growing in favor among the men, perhaps because of "propaganda", and may account for some of the delay in return of gum disease, even though it does not supply enough vitamin C to maintain good plasma levels. Five of the thirty-four subjects last examined had also been getting extra fruit, but in small quantities. Their plasma readings were between 0.2 and 0.4 %. Three others were taking vitamin pills, also in insufficient amounts. Their plasma readings were 0.59, 0.23 and 0 mg. %.

Consideration was given to the relationship between the severity of oral disease and age; also to the length of prison confinement (table 2). It was observed that the preponderance of severe disease is in the older group and also in those with the longest prison sentences.

Oral hygiene was noted for its possible connection with the prevalence of gum disorders. The first inspection showed that some men were regular in the care of their teeth. The prisoners are supplied with tooth

brushes and tooth powder for daily use. A full time dentist is employed by the prison. Many of them had made efforts to improve their gum conditions and were concerned over the necessity for frequent extractions. We therefore think that poor oral hygiene is not the major factor in the frequent severe gum disease seen in these prisoners. Prison diet seems to be the one constant factor which is responsible for high incidence of oral disease among the inmates. In every case where the diet was unsupplemented by extra fruit there was moderate to severe damage if the prison stay had been longer than 2 years. This

TABLE 2
Incidence of oral disease.

ORAL DISEASE	NO.	AGE GROUP		LENGTH OF PRISON CONFINEMENT, YEARS				REMARKS
		Over 30	Under 30	Over 10	5-10	2-5	1-2	
Severe	30	27	3	6	10	11	3	16 had no extra fruit
Moderate	14	6	8	..	3	4	7	2 had no extra fruit
Slight	27	11	16	..	3	12	12	3 had no extra fruit

was particularly significant in several of the younger subjects (less than 25 years old) whose teeth were excellent and mouths very clean but whose gums were reddened and edematous and bled at the least provocation.

DISCUSSION

As early as 1930 Hanke reported on the relationship between vitamin C deficiency and disease of the oral tissues. He described the conditions affecting the gums that were consistently observed in this study. His method of increasing the ascorbic acid intake was by feeding $\frac{1}{2}$ to 1 pint of orange juice plus the juice of one half a lemon daily and including large quantities of raw cabbage or lettuce in the diet. We observed the same type of healing and gum improvement with the synthetic ascorbic acid which we gave in smaller doses over a longer period of time. Hanke's dosage probably furnished between 100 and 300 mg. of vitamin C from his daily prescribed fruit juices. Our primary interest was to find out how small a continuous dose would maintain the oral tissues in normal condition, since the larger doses may not now be obtainable, and should in any case, of course, not be wasted.

Tissue changes in vitamin C deficiency occur first in the supporting tissues of the teeth. Later changes affect the other supporting tissues of the body and are evidenced by slow healing of lesions and later by joint and bone changes. An adequate review of this subject has been presented by Wolbach and Bessey ('42). In our observations it was noted that among the large number of men with marked oral disease, accompanied by zero plasma ascorbic acid, there were several who participated in strenuous sports like wrestling and baseball. This was in addition to their regular work in the prison machine shop, twine plant, laundry, etc. All the men chosen were able to work steadily and were of the healthiest type of prisoner. The deficiency effects observed in oral tissues would appear to antedate practically any other detectable physical change from the normal.

Since the plasma is merely the vehicle by which the vitamin is distributed, the plasma values are the first to fall in deficiency states, but the last to return to normal when the tissues are resupplied. It has, therefore, been argued that a more quantitative index of severer degrees of deficiencies would be a measurement of the vitamin in blood cells, or whole blood that includes the cells. So far, only the plasma determination is technically practicable for survey studies like the present one. While it cannot be relied upon to differentiate among the severer degrees of tissue desaturation, we can certainly be safe in inferring from the very low plasma values found so consistently in our untreated subjects that supplies to the tissues were far from adequate. This is supported by the now widely accepted fact that in well nourished individuals plasma ascorbic acid concentrations are found nearly always above 0.7 or 0.8 mg. %, while under dietary conditions called optimal, and in the absence of illness or infection, levels in the plasma remain usually above 1.0 mg. %. The agreement between plasma ascorbic acid level and gum changes was so close throughout this study that it seems justifiable to use the plasma readings, or better, their response to added vitamin C as a criterion for determining the minimal needs.

In following out the separate curves for each of the men on the different supplements, it became apparent that ingestion of 50 mg. a day could not be called adequate. On the regular prison diet, entirely un-supplemented, we found certain controls whose plasma ascorbic acid stayed at zero the year round. Those controls who showed a slight seasonal rise were able to obtain some extra fruit at that time. A 3-year study of an institutional diet by Horwitt ('42) included plasma ascorbic acid studies on 380 "average patients" in a hospital for the insane. He

estimated that their daily diet included not more than 25 mg. daily. The lowest average figure for plasma ascorbic acid (0.2 mg. %) was found during the months of March to June. The highest average value occurred in October and November (0.59 mg. %) while the over all average was less than 0.4 mg. %. Barahal and Priestman ('42) reported 0.419 mg. % as the average plasma ascorbic acid in a group of forty-four mental patients who were maintained on an unsupplemented hospital diet over a long period of time. They state that "a definite correlation has been found between the frequency of dental and gingival conditions in mental patients and their ascorbic acid levels".

A detailed study of the vitamin C requirements of six healthy female individuals was made by Storwick and Hauck ('42). They conclude that to maintain tissue saturation 65 to 150 mg. of ascorbic acid must be given in addition to approximately 10 mg. in the diet. They pointed out, as have others, wide individual variations. Fincke and Landquist ('42) set 0.8 mg. % as their standard for normal plasma ascorbic acid level. They found it required 0.8-1.2 mg. of ascorbic acid per kilo. body weight per day to maintain this level. This individual variation in vitamin C needs is becoming generally recognized. In such a group as we have studied, conditions outside the individual physiology were uncommonly standardized in comparison with those ordinarily obtainable in a similar-sized healthy group. Variations became apparent in the response of both blood levels and health of oral tissues to added amounts of vitamin C.

CONCLUSIONS

The probable minimal daily amount of vitamin C needed by healthy male adults lies in the neighborhood of 75 mg., as gauged by the response of the plasma ascorbic acid values and health of the gum tissues. Refractory individuals with low grade chronic infections such as nose and throat infections or deeply diseased gums appear to need at least 100 mg. daily to obtain a satisfactory response.

There appears to be a correlation between the duration of vitamin C deficiency and the severity of oral disease.

Ascorbic acid in institutional diets should be provided for by citrus and other fresh fruits, by increased amounts of tomatoes either fresh or canned (but not reheated), by supplements of synthetic ascorbic acid, or by a combination of these.

SUMMARY

The daily vitamin C requirements of seventy-one male prisoners were investigated over a period of 17 months.

Oral disease was prevalent among the younger as well as the older subjects.

The daily food lists revealed that no fresh fruit and very few fresh vegetables were included in the diet.

Laboratory results of plasma ascorbic acid determinations in untreated individuals showed that only a very few men, who were securing oranges or vitamin preparations for themselves, had plasma values over 0.6 mg. %; nearly 80% were under 0.2 mg. %, and twenty out of seventy-one showed no ascorbic acid in the plasma. Figures were very slightly higher in August and September than during the rest of the year.

Results of ascorbic acid supplements on plasma and gums were as follows: 25 mg. daily gave no significant elevation of the plasma values and only slight improvement in the gum disease; 50 mg. raised the plasma values noticeably, and definite improvement was noted in the gums, but this amount was not sufficient to maintain a plasma level of 0.8 mg. % during the winter and spring months. With a 75 or 100 mg. supplement the most satisfactory response of both plasma and gum healing was obtained. The 75 mg. also appeared sufficient for most of the men throughout the year. The few cases refractory to 75 mg. responded to an increase in dosage (100 mg.) within 4 to 5 weeks.

ACKNOWLEDGMENT

It is a pleasure to record our gratitude for the cooperation of a number of individuals. Mr. Albert Bailey, Secretary of the Wisconsin Board of Public Welfare, made arrangements authorizing the study in the State Institution. The enthusiastic cooperation of Mr. J. C. Burke, warden, shown by his presentation of the idea to the men and his readiness to advise with us about procedures, was an indispensable factor in the success of the study. We have enjoyed, on every occasion when we have requested it, the assistance of Dr. A. J. Hebenstreit, the physician to the prison; Dr. T. F. Meagher, the dentist; and Mr. I. C. Breitlow, the head nurse. The prison chef, Mr. Casper, has been very helpful. We are glad to acknowledge the sustained cooperation and dependability in reporting details of the participating inmates of the prison throughout the investigation.

The counsel and critical advice of Dr. C. A. Elvehjem and Dr. H. T. Parsons are sincerely appreciated.

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THE EFFECTS ON RESPIRATORY METABOLISM PRODUCED BY EQUAL AMOUNTS OF CAFFEINE IN THE FORM OF COFFEE, TEA AND THE PURE ALKALOID

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ONE FIGURE

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The present experiments were undertaken to determine whether caffeine in coffee or in tea differs in its effect on the respiratory metabolism from the same amount of the pure caffeine alkaloid in solution. In a study of reaction time as influenced by caffeine, Cheney ('35) found that coffee produced results parallel to those obtained from capsulated caffeine but to a lesser degree. Large doses of caffeine always led to a decrease in the reaction time but the maximum reduction was usually greater with pure caffeine than with an equivalent amount in the form of coffee as a beverage. These results would suggest that the caffeine in coffee may be absorbed more slowly or to a lesser extent than from an alkaloid solution. If there are any differences in the rate or extent of absorption they should be reflected in the respiratory metabolism, which is definitely affected by caffeine (Haldi, Bachmann, Ensor, and Wynn, '41).

METHOD

The procedure was essentially the same as in our previous experiments ('41) on the respiratory exchange following the ingestion of caffeine alkaloid. Immediately after the usual preliminary basal period, the subject drank 200 ml. of a fresh infusion of coffee or tea at 37°C. or an equal volume of water containing caffeine alkaloid in solution. The same packages of a standard brand of coffee and of tea were used throughout the experiment. Ten grams of tea were added to 220 ml. boiling water, stirred for 1 minute and allowed to stand for 9 minutes. As much of the supernatant tea as could be recovered was poured off and made up to exactly 200 ml. Analysis of eight different infusions gave an average caffeine content of 195 mg. in 200 ml. tea with an average deviation of 6 mg. A coffee infusion made in the same

way from 25 gm. of coffee contained an average of 204 mg. caffeine. For the caffeine alkaloid solution 200 mg. pure caffeine was dissolved in 200 ml. water. The unavoidable difference of several milligrams caffeine in the coffee and tea infusions and in the caffeine solution was regarded as of no consequence inasmuch as it had been found in the former experiments that 60 mg. caffeine had no effect on the respiratory metabolism. Two minutes after the ingestion the subject reclined on a couch and began breathing through the apparatus immediately. The gaseous exchange was determined at intervals of 15 minutes for a period of 1 hour and 45 minutes.

The subjects were well trained for this type of experiment, having taken part in several hundred other experiments of a similar nature. More reliable results, we believe, can be obtained from one or two subjects with adequate training than by taking averages of data derived from a large number of untrained subjects. As we have had occasion to observe before, errors may be introduced either by restlessness, or dozing or incomplete relaxation on the part of the subject, and in our experience, careful selection of the subjects and sufficient training are essential prerequisites for reliability of the experimental data.

Since it is possible that any effect produced on the respiratory exchange by coffee and tea might be due in part to other extractives than caffeine, a series of experiments were run as controls in which decaffeinated coffee was ingested. Analysis showed that the caffeine content was negligible. Eight experiments were done on each subject with each beverage.

RESULTS

Oxygen consumption

A dose of 0.2 gm. caffeine alkaloid had a definite stimulating action on oxidative metabolism as shown in the curves of oxygen consumption in figure 1. The rate of oxidation rose shortly after ingestion of the alkaloid and reached a peak (14.3% above the basal) during the first 15-minute period in one subject and within 30 minutes in the other (12.9% above the basal). This was followed by a gradual decline. At the conclusion of the experiment, however, the oxygen consumption had not returned to the basal level. These results are in conformity with those obtained in a previous study on caffeine (Haldi et al., '41).

Comparison of the curves in figure 1 shows that the stimulation of metabolism by coffee and tea was practically the same as that produced by the alkaloid. The small differences observed in some of the individual periods were within the normal range of variation. The total

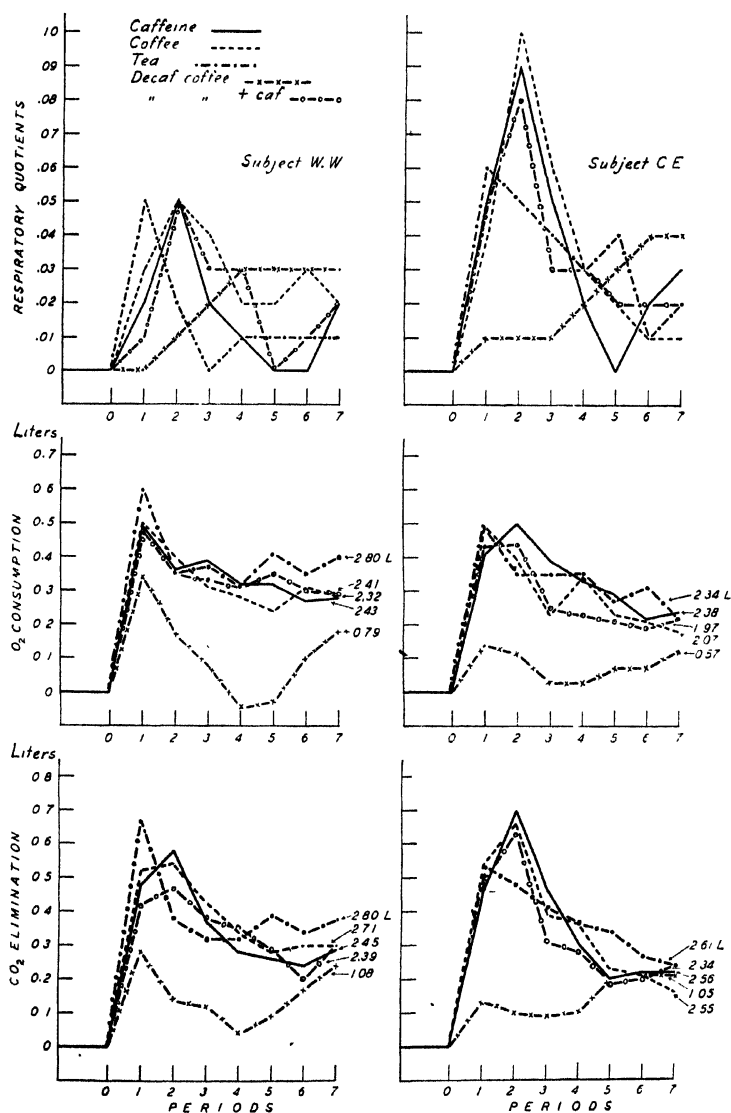


Fig. 1 The course of respiratory metabolism with reference to basal conditions following the ingestion of various beverages containing caffeine or decaffeinated coffee. The total excess metabolism above basal is indicated at the end of each curve.

increase in oxygen consumption above the basal for the entire experiment is given in the right hand margin of the curves in figure 1. The differences in the increase in oxygen consumption after taking tea, coffee, or the alkaloid were not statistically significant.

That the stimulating effect of coffee on oxidation was due to the caffeine content is evident from the experiments in which decaffeinated coffee with caffeine added was ingested. The increase in oxidation in these experiments (fig. 1) was approximately the same as that induced by coffee. These results are in agreement with those reported by Horst, Willson, and Smith ('36). The increase in oxidation after taking decaffeinated coffee was the same as has been obtained in numerous other experiments on our subjects following the ingestion of the same amount of water. Although no experiments were conducted with decaffeinated tea, it may be concluded in view of the comparative effects of coffee and decaffeinated coffee that the stimulation of oxidative metabolism by tea was due to its caffeine content.

Carbon dioxide elimination

The carbon dioxide elimination, like the oxygen consumption, rose promptly after the ingestion of coffee, tea and caffeine alkaloid, reaching a peak within 15 or 30 minutes after ingestion. The differences in the individual periods (fig. 1) were of no statistical significance; likewise the total increase above the basal for the entire experiment shown on the right of the curves was not significantly different in the three groups of experiments on either of the subjects. As in the case of oxygen consumption the increase in carbon dioxide elimination after taking coffee must be attributed to the caffeine content. The ingestion of decaffeinated coffee produced approximately the same effect as that previously observed after taking water, whereas when caffeine was added to decaffeinated coffee the effect was the same as that obtained with coffee.

Respiratory quotient

The curves of the respiratory quotients of the two subjects, shown in figure 1, present the same general features throughout the experiment. Ingestion of coffee, tea and caffeine alkaloid was followed by a prompt rise in the quotient of both subjects during the first 15-minute period, which continued and reached its peak in the second period, except in the experiments with tea on W. W. In these the peak of the rise was attained in the first 15-minute period. The quotient of C. E. rose more

abruptly in the first period and reached a greater height in the second period than in the case of W. W. Similar differences in the respiratory quotient of these two subjects after the ingestion of caffeine had been observed in earlier experiments ('41).

While there were some differences in the rise of the quotient above the basal in the various 15-minute periods after taking coffee, tea and alkaloid, the total effect on the quotient for the entire duration of the experiment was the same for the different groups of experiments. The respiratory quotient for 105 minutes after ingestion (seven 15-minute periods) in each group was 0.03 above the basal in one subject and 0.02 above in the other.

In the control experiments with decaffeinated coffee the quotients were practically the same as in numerous other experiments, not included in the present study, in which water alone was taken. The rise in the quotient of C. E. toward the latter part of the control experiments with decaffeinated coffee (which was the usual occurrence also in the experiments in which water alone was ingested) was probably due to a slight degree of restlessness because of the discomfort of lying still and breathing continuously through the mouthpiece.

Interpretation of the respiratory quotient

The effect of coffee and of tea on the respiratory quotient must be attributed solely to their caffeine content. This becomes obvious upon comparing the action of caffeine alkaloid with that of decaffeinated coffee with and without the addition of caffeine.

The interpretation offered for the behaviour of the quotient after the ingestion of caffeine in a previous study ('41) applies to the present experiments with coffee and tea. Carbohydrate oxidation was not increased, as is evident from the slight extent of the rise in the quotient above the basal for the entire experiment. This small rise of 0.03 and 0.02 was no greater than that which occurred in the control experiments with decaffeinated coffee and in previous experiments in which water alone was taken. For the same reasons adduced in our previous study on caffeine, it may be concluded that the marked rise in the quotient after taking coffee and tea was due to the hyperventilation caused by the caffeine content. The hyperventilation was a consequence of an increase in respiration and was completely compensated by retention of carbon dioxide before the conclusion of the experiment. (Cf. Haldi, Bachmann, Ensor and Wynn, '41).

Respiratory ventilation

The effects of coffee and tea on respiratory ventilation as shown in table 1, were the same as those produced by caffeine in solution. The marked rise in the minute volume of inspired air following the ingestion of the caffeine solution was similar to that obtained with the alkaloid in previous experiments ('41). There was a small increase in the experiments with decaffeinated coffee but this was practically the same as that which we have observed after the ingestion of the same amount of water.

TABLE 1

Respiratory ventilation (liters per minute at 0°C. and 760 mm. Hg.) as affected by caffeine taken in the form of alkaloid, coffee, and tea.

Subject — W. W.

	BASAL	CONSECUTIVE 15-MINUTE PERIODS							AVG. INCREASE PER MIN.
		1	2	3	4	5	6	7	
Caffeine alkaloid	4.80	+1.02	+1.10	+ .90	+ .81	+ .72	+ .60	+ .77	.85
Coffee	4.84	+1.06	+1.13	+1.02	+ .90	+ .72	+ .83	+ .88	.93
Tea	4.90	+1.29	+ .94	+ .86	+ .79	+ .98	+ .86	+ .98	.96
Decaffeinated coffee	4.52	+ .52	+ .23	+ .09	— .05	+ .14	+ .23	+ .41	.22
Decaffeinated coffee and caffeine	4.46	+1.01	+1.26	+1.00	+ .88	+ .84	+ .71	+ .80	.93

Subject — C. E.

Caffeine alkaloid	5.30	+ .73	+1.24	+ .92	+ .81	+ .63	+ .56	+ .52	.77
Coffee	5.00	+ .86	+1.18	+ .84	+ .69	+ .59	+ .64	+ .68	.78
Tea	5.22	+1.13	+ .77	+ .93	+ .91	+ .87	+ .87	+ .72	.89
Decaffeinated coffee	5.10	+ .38	+ .09	+ .05	+ .08	+ .24	+ .25	+ .33	.20
Decaffeinated coffee and caffeine	5.23	+ .99	+1.30	+ .83	+ .74	+ .54	+ .46	+ .60	.78

It may therefore be concluded that the stimulating action of coffee and tea on respiratory ventilation was due solely to the caffeine content of these beverages.

SUMMARY AND CONCLUSIONS

A comparative study has been made of the gaseous exchange of two well trained subjects following the ingestion of 200 mg. caffeine alkaloid and of coffee and tea infusions containing this amount of caffeine.

The action of caffeine on oxygen consumption, carbon dioxide elimination, the respiratory quotient and respiratory ventilation was the same as that observed in previous experiments of this kind. Coffee and tea had the same effect as the alkaloid.

Comparison of these results with those obtained with decaffeinated coffee and decaffeinated coffee to which caffeine was added shows that the effect of coffee and tea was due solely to their caffeine content.

It may be concluded from these experiments that the rate of absorption and the action of caffeine in coffee and tea is the same as that of the pure alkaloid in solution.

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SOME OBSERVATIONS OF DARK ADAPTATION IN MAN AND THEIR BEARING ON THE PROBLEM OF HUMAN REQUIREMENT FOR VITAMIN A ¹

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ONE FIGURE

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INTRODUCTION

In a previous paper, Batchelder and Ebbs ('42) reported the results of a study on the dark adaptation of college students and its relation to vitamin A metabolism. Some preliminary studies of vitamin A requirement were included which indicated that in three young women 5500 I. U. vitamin A was slightly more than their minimum requirement. The purpose of the work described below was to throw more light on the question of minimal needs for vitamin A. The few figures on requirements now found in the literature show for the most part the amount of vitamin A (or plant precursors) required to restore to normal an impaired visual threshold. Under such conditions some of the vitamin A might actually be used in repair during recovery and in storage, after the lowest threshold attainable by the individual has been reached. Such dietary requirement figures for vitamin A might therefore be expected to be somewhat higher than the minimal because storage could conceivably account for a fraction of the vitamin A intake. The more rapid the rate at which the visual threshold approaches the individual's optimum, the more likely it is that the vitamin A intake is exceeding the minimal requirement for maintaining that threshold. It seemed desirable therefore to approach the question of minimal dietary requirements from another standpoint in order to eliminate storage as a factor. The maintenance of visual thresholds somewhat above those shown by the subjects before changing to a vitamin A deficient diet has been studied for that purpose. The results are

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here reported along with additional information on (1) time required on a depletion diet for onset of impaired visual sensitivity, (2) rate of rise in the visual threshold when depleted subjects were continued on a diet practically devoid of vitamin A value, (3) changes in the character of the dark adaptation curve as visual sensitivity decreased, and (4) return of depleted subjects to normal.

The results of the present experiment are not necessarily to be interpreted as indicating a practical dietary requirement (minimal vitamin A needed to support maximal visual sensitivity). The maintenance figures present values lower than the amounts which could apparently be used to advantage by the individuals studied.

EXPERIMENTAL

Technique

The instrument used for the measurement of dark adaptation was the Rhodometer (Batchelder and Ebbs, '42). The standard procedure previously used in survey tests establishing a normal range for several hundred college students was followed using both the 30- and the 15-minute thresholds as criteria of ability to adapt to darkness. The procedure involved light adaptation of 3 minutes immediately preceding the measurement of rate of dark adaptation. The minimum brightness perceived at different times during the dark adaptation was recorded and plotted as the logarithm of brightness in micromicrolamberts. An artificial pupil was used at all times.

Procedure

Three female subjects, M. W., E. G., and Y. H., and one male, R. Y., volunteered to go on a vitamin A-low diet. All underwent physical and dental examinations which showed them to be healthy young adults.

The subjects were placed on a diet calculated to yield below 200 I. U. of vitamin A per day. The selection of the foods low or lacking in vitamin A was made through the use of the tables of Daniel and Munsell ('37), Munsell ('40), and Booher and Marsh ('41).

The dark adaptation of each subject was measured at least three times weekly and practically daily during the time vitamin A supplements were fed.

The four subjects ate meals prepared in the laboratory kitchen and were served approximately uniform portions with the exception of vitamin A-free breads and sweets which were taken at will. All received 50 mg. of crystalline ascorbic acid plus 4 oz. of canned grapefruit

juice daily. Activated ergosterol was administered weekly in doses of 3,000 to 4,000 I. U. In addition, each subject received 6 brewers' yeast tablets daily. One pint of skimmed milk (0.02% butterfat) was consumed daily by each subject. The nature of the ergosterol capsules was unknown to the subjects who suspected vitamin A. Moreover, when vitamin A feeding was begun, all subjects received either placebos or supplements.

RESULTS

Time elapsing before signs of poor dark adaptation appear

Chart 1 shows the 30-minute threshold for each subject. A trend toward higher visual thresholds was observed in these subjects although in subject Y. H., an abrupt drop occurred on the eighty-fifth day and a low threshold was maintained from then to the end of the experiment. Subjects R. Y., M. W., and E. G. showed definite rise in visual threshold within the range of 16 to 124 days on the depletion diet as reported by Booher et al. ('39). The record of Y. H. is somewhat similar to that reported by Steffens et al. ('39), and Brenner and Roberts ('43), who reported that only slight and temporary rises in threshold were observed in subjects on a vitamin A-deficient diet for 6 months. None of our subjects showed the immediate rise in threshold following a vitamin A-free regime reported by Hecht and Mandelbaum ('40). Blanchard et al., ('40) reported that 10 to 31 days were necessary, and Batchelder and Ebbs ('42) found that 29 to 73 days on the diet were required before the threshold rose.

Rate of depletion and response to vitamin A supplements

All four subjects showed a sensitivity well within the normal range (Batchelder and Ebbs, '42) at the beginning of the experimental period. The rate of change in visual sensitivity varied among the four subjects.

Subject E. G. showed a markedly higher threshold on the eighty-first day, but otherwise showed only a slight, steady rise amounting to about 0.5-log unit by the one hundred and eighteenth day. This was not arrested by the feeding of 2,000 I. U. of vitamin A concentrate daily from the one hundred and eighteenth to the one hundred and forty-fifth day. The feeding of 4,000 I. U. daily (84 I. U. per kilogram of body weight) resulted in a drop in the threshold to a level similar to that which was shown at the beginning of the 2,000 I. U. feeding period. This level was 0.7- to 0.8-log unit above her threshold at the beginning of the experiment, and was maintained with few exceptions from the one hundred and forty-fifth to the one hundred and eightieth day on an intake of 4,000 I. U. of vitamin A daily.

Subject M. W. showed a marked rise in threshold between the seventy-fourth and seventy-sixth day, at which point it remained constant for about 10 days and then increased on the eighty-ninth day to approximately 1.0-log unit above her original threshold. The feeding of 2,000

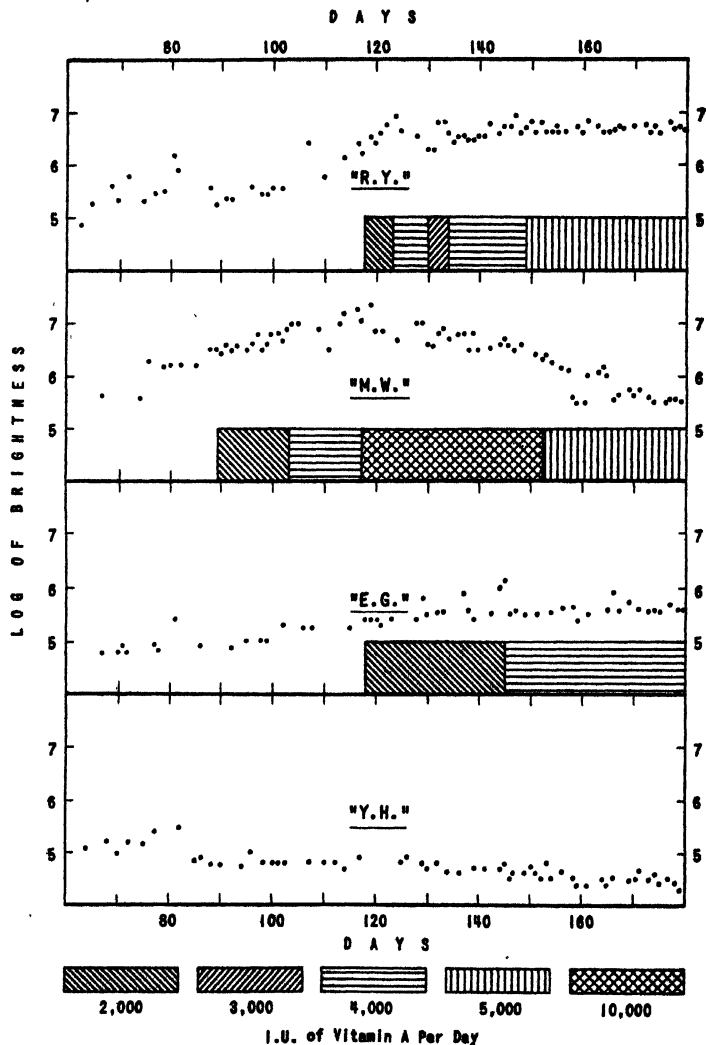


Fig. 1 Thirty-minute thresholds for perception of brightness at different times following a change from a freely chosen to a vitamin A-deficient diet and after feeding known amounts of vitamin A.

I. U. daily failed to check this rise. Blood vitamin A and blood carotene were within the normal range, 620 and 440 I. U. per 100 ml., respectively, on the ninety-ninth and one hundred and fourth day. In spite of the apparently normal blood picture, it was decided to increase the vitamin A supplement to 4,000 I. U. daily. The threshold dropped during the next 6 days but by the seventeenth day was higher than before (1.7-log units above the original level). On the one hundred and eighteenth day, the subject was emotionally unstable, and although she was unaware of the results of the tests, reported that she felt "helpless" and "all gone."

The vitamin A supplement was increased to 10,000 I. U. until the threshold reached the level at which the first feeding tests of the vitamin A concentrate were started. This level was reached on the one hundred and fifty-second day, at which time the feeding of 5,000 I. U. daily was begun. The threshold continued to drop for several days, probably due to residual stores from the previous period. From the one hundred and sixty-fifth day to the one hundred and eightieth day, the threshold was close to the subject's original dark-adaptation level. If the effect of previous higher feeding can be ruled out, this represented maintenance of a normal dark-adaptation threshold on an intake of 81 I. U. per kilogram of body weight. Return to a freely-chosen diet plus 10,000 I. U. of vitamin A daily for 45 days caused no further improvement in her threshold.

Subject R. Y. showed a rise in threshold from the sixty-fifth to the eighty-first day which was followed by a spontaneous drop which reached its lowest point on the eighty-ninth day. This was similar to the drop observed at about the same time in subject Y. H. and also less definitely in the record of E. G. A fairly steady increase in threshold followed in the case of R. Y. until, on the one hundred and seventeenth day, it was 1.5-log units above the normal level. A supplement of 2,000 I. U. daily for 6 days failed to check this increase, and 4,000 I. U. were fed. This resulted in a marked drop in the threshold in 7 days, at which point 3,000 I. U. were tried for 4 days. The threshold rose again, however, and the 4,000 I. U. level was resumed. A slow increase was evident after the one hundred and first day, and on the one hundred and forty-ninth day, the supplement was raised to 5,000 I. U. At this level of intake of vitamin A the dark-adaptation ability remained very constant to the one hundred and eightieth day. This, however, was a level almost 2.0-log units above the subject's normal dark adaptation level. At that date, the controlled vitamin A-deficient diet had to be discontinued and it was not possible to determine how

much was necessary in order to maintain his dark adaptation at a more favorable level. The 5,000 I. U. intake level represented 74 I. U. per kilogram and is inadequate for the maintenance of maximal dark adaptation for this subject. After the one hundred and eightieth day, he returned to a freely-chosen diet plus approximately 10,000 I. U. of vitamin A daily. Recovery was slow, however, and after 42 days he was given 300,000 I. U. daily. This resulted in a sharp drop. Subsequent feedings of this amount for 9 more days resulted in only slow recovery. The initial sharp drop following the first massive dose appears in line with our own previously reported experience and with that of several others cited by Rajogopal ('41).

Character of the dark-adaptation curve

During the early stages of depletion (when 30-minute dark adaptation thresholds were rising) the 15-minute thresholds tended also to rise, but the earlier thresholds, where cone vision was apparently functioning, did not rise until later in the depletion period.

DISCUSSION

The difficulties of presenting the results of dark adaptation tests in the form of tables have made it possible to present in detail only the 30-minute thresholds of the subjects during the part of the experimental period beginning just before the first significant change in dark-adaptation ability was observed. Chart 1 presents these data. It will be observed that after a period of about 2 months, during which the effect of the vitamin A-deficient diet was apparently compensated for by bodily stores of vitamin A, a rise in the thresholds occurred. The initial rise, which is interpreted as indicating that bodily stores have been depleted, was followed by a decided drop in three of the four subjects. This drop, in the case of E. G. and R. Y. was followed by a second gradual increase. The drop might be interpreted as a temporary adjustment of the body to a lower level of vitamin A metabolism, which could not be maintained in the face of a persistent shortage. In the case of Y. H., however, a second increase did not follow. The fourth subject, M. W., showed no temporary arrest in the trend toward poorer dark adaptation. Her response to the different levels of feeding chosen was very consistent. The fact that her dark-adapting ability appeared to improve for a considerable period after being changed from a 10,000 to a 5,000 I. U. supplement is interpreted as indicating that some storage of vitamin A had been possible.

The variation in the rate of depletion of different subjects may be partly due to individual differences in physiologic response to vitamin A deficiency, as in the case of one of our subjects and a few others reported in the literature who have failed to show a decrease in dark adaptation after many months on a vitamin A-deficient diet. Unusually large body stores of the vitamin, or more economical use of them are probably the chief reason for lack of evidence of deficiency after many months on a deficient diet.

SUMMARY AND CONCLUSIONS

Four young adults were fed on a diet deficient in vitamin A but adequate in all other known nutrients. Their dark adaptation was measured by means of the Rhodometer, previously described. One subject showed slight initial rise in visual threshold, but after the eighty-fifth day, showed a gradual improvement in dark adaptation up to the one hundred and eightieth day when the study was discontinued. The others showed a rise in visual threshold after 65 to 100 days. Certain aspects of the rate of change in visual threshold have been brought out. When the rise amounted to about 0.5-log unit, one subject was fed 2,000 I. U. of vitamin A daily for 27 days. Since the threshold continued to rise, the dose was increased to 4,000 I. U. This resulted in maintenance of a constant 30-minute threshold for about 45 days. Two subjects were treated in a similar manner except that the visual threshold was allowed to rise 1.0-log unit before vitamin A was fed. In both instances, 4,000 I. U. were inadequate for maintenance, but 5,000 I. U. served to maintain constant but subnormal dark adaptation for about 1 month. Individual differences in response to large doses of vitamin A and in return to normal visual sensitivity were observed.

In three different subjects, 74 I. U. vitamin A per kilogram body weight (5,000 I. U. per day) sufficed only to maintain a threshold 1.5-log units above normal; 84 I. U. per kilogram (4,000 per day) maintained a threshold 0.5-log units above normal; and 81 I. U. per kilogram (5,000 I. U. per day) maintained an approximately normal threshold.

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A STUDY OF NEUROMUSCULAR REGENERATION UNDER DIFFERENT LEVELS OF VITAMIN C INTAKES¹

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Numerous reports have appeared which indicate the importance of an adequate intake of vitamin C for the proper healing of surgical wounds in man and the guinea pig. Information is relatively meager concerning the role of vitamin C in the regeneration of muscle and nerve tissues. Dalldorf ('29) reported that severe scurvy in experimental animals was accompanied by extensive muscle damage. Hojer ('24) considered such muscle lesions to be characteristic of scurvy. Kodicek and Murray ('43) noted that guinea pigs recovering from injury to bone and muscle on diets partially deficient in vitamin C exhibited muscle swelling and overgrowth of connective tissue. These investigators recommended a liberal supply of vitamin C to patients recovering from injury to bones and muscles.

The present investigation was designed to study the relation of vitamin C intake to muscle and nerve regeneration. Information was sought concerning the effects of suboptimal intakes and excess intakes of ascorbic acid upon the extent and velocity of muscle and nerve regeneration following peripheral nerve injury.

EXPERIMENTAL

A total of fifty young guinea pigs were selected from a common stock, matched as to age, body weight and sex and equally divided among five groups. The basal ration (Eddy, '29) used throughout these studies had the following percentage composition: baked skim milk powder, 30; butterfat, 9; sodium chloride, 1; rolled oats and bran (equal parts by volume), 59; and cod liver oil, 1. The above diet was supplemented with 1.5 mg. of alpha-tocopherol² per animal per day and with ascorbic acid in amounts of either 0.5, 1.0, 2.5, 5.0 or 50.0 mg.

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² The alpha-tocopherol was supplied through the courtesy of Hoffman-LaRoche, Inc., Nutley, N. J., and Merck and Company, Rahway, N. J.

per day. The vitamin supplements were administered orally from a pipette. A few days were allowed for the animals to become accustomed to their diets before operation.

A complete denervation of one gastrocnemius muscle was accomplished by exposing the tibial nerve under ether anesthesia and crushing it with a heavy linen ligature against a brass rod. This procedure allowed for complete denervation and preserved a good alignment for subsequent nerve regeneration. The unoperated contralateral muscle and nerve served as controls. Studies were made upon the muscles and nerves of the operated and control limbs at 35 days after denervation. At this time the regenerating nerves had established functional contacts with their muscles and the latter were recovering mass and strength which had been lost prior to reinnervation.

Measurements were made concerning the strength of the regenerating and contralateral control muscles. This was done by measuring the maximal isometric tension response to volleys of slightly supermaximal stimuli applied directly to the muscle and to its motor nerve. These operations were carried out under light ether anesthesia. The techniques employed for stimulation and strength measurements have been described in detail elsewhere (Hines, Thomson and Lazere, '42). It is to be noted that under these conditions the tension responses of control non-denervated muscles to nerve stimulation were of the same magnitude as those elicited by direct muscle activation. However, in the case of muscles undergoing reinnervation and regeneration, the tension responses of the muscles to nerve stimulation were only a fraction of those developed in response to direct activation. These techniques made it possible to determine quantitatively the extent of functional motor reinnervation as well as the total strength of the muscles of guinea pigs subsisting on different levels of vitamin C intakes. At the conclusion of the tension measurements, the gastrocnemii were carefully dissected and weighed in order to determine the degree of atrophy present at that time.

The experiments described above were not carried out on animals subsisting on less than 0.5 mg. of ascorbic acid per day, inasmuch as their survival time would have been too short and uncertain to permit comparable times for studies upon regeneration. In other experiments determinations were made of the strength, water, chloride and creatine concentration of gastrocnemius muscles from guinea pigs subsisting for 20 days on the basal C-deficient diet without supplements and from control animals whose diet was supplemented with 10 mg. of ascorbic acid per day.

EXPERIMENTAL RESULTS

A summary of the average values obtained from a study of forty-three animals appears in table 1. It is to be noted that non-denervated gastrocnemii from animals subsisting on different intakes of ascorbic acid, ranging from 0.5 to 50.0 mg. per day, exhibited essentially the same tension per unit of muscle weight. This finding was true for the responses to nerve stimulation as well as to direct muscle activation. The muscles of the control limbs appeared normal grossly and capillary hemorrhages were not observed. However, the non-denervated gastrocnemius muscles of scorbutic animals which had subsisted on a

TABLE 1

A summary of average values together with standard errors for the regenerating and control gastrocnemius muscles and tibial nerves of guinea pigs on different levels of ascorbic acid intakes. The measurements were made 35 days after a unilateral crushing of the tibial nerve.

NO. OF ANIMALS	DAILY INTAKE OF ASCORBIC ACID	TENSION PER GRAM MUSCLE WHEN ACTIVATED THROUGH				RELATIVE ¹ STRENGTH OF REGENERATING MUSCLE WHEN ACTIVATED THROUGH		RELATIVE ¹ WEIGHT OF REGENERATING MUSCLE
		Nerve		Muscle		Nerve	Muscle	
		Exper.	Control	Exper.	Control			
	mg.	gm.	gm.	gm.	gm.	%	%	%
9	0.5	368±37	1523±113	477±72	1533±174	18.9±4.9	29.8±7.4	85.8±6.9
10	1.0	515±57	1651±47	799±51	1652±56	28.2±2.6	44.7±3.6	91.3±4.1
8	2.5	780±39	1371±77	1191±30	1540±72	51.4±3.9	66.8±3.6	81.9±1.6
7	5.0	890±44	1581±100	1198±75	1660±94	45.2±4.9	57.9±5.7	82.1±2.4
9	50.0	945±53	1562±69	1117±39	1642±69	50.5±2.0	56.9±1.5	83.6±2.0

¹ Expressed as percent of values for non-denervated contralateral control.

C-deficient diet for 3 weeks were found to be weaker than comparable muscles of animals which had received adequate intakes of this vitamin (table 2).

Essentially the same degree of atrophy and strength per unit of weight was found for the regenerating gastrocnemius muscles of guinea pigs subsisting on a daily intake of 2.5 mg. as in the experiments with intakes as high as 50.0 mg. of ascorbic acid per day. The ratios of the tensions developed by the experimental muscles to that by their non-denervated controls were not affected by these different levels of vitamin C intakes. This was true for both the tensions elicited by nerve stimulation and by direct muscle activation. However, the tensions per unit weight for the regenerating muscles of animals receiving either 0.5 or 1.0 mg. of vitamin were consistently lower than those receiving the larger intakes. Likewise, the ratios of the tensions

developed by the regenerating muscles to those by their contralateral controls were lower than those observed for animals subsisting on ascorbic acid intakes usually regarded as optimal or excessive. The findings of lowered ratios for tension development for both nerve and direct stimulation indicate that subnormal intakes of ascorbic acid lead to impaired functional regeneration of muscle and nerve. The data (table 1) indicate that the extent of atrophy, when calculated on a wet weight basis, was less on the low ascorbic acid intake than on the higher levels of intake. Calculations of atrophy from differences in wet muscle weights may be misleading because the regenerating muscles of the animals on the lower ascorbic acid intakes exhibited numerous capillary hemorrhages which were not observed in their non-denervated contralateral controls. Hemorrhagic lesions were absent from both the control and regenerating muscles of animals receiving optimal or above

TABLE 2

A summary of average values for the gastrocnemii of guinea pigs on vitamin C-free diets and control diets with adequate intake of C.

DIET	NO. OF ANIMALS	TENSION PER GM. MUSCLE	CREATINE PER 100 GM. DRY WEIGHT MUSCLE	MUSCLE WATER	CHLORIDE PER 100 GM. MUSCLE
		gm.	mg.	%	millimols
Control	6	1703	2032	76.90	49.6
C-free	6	1404	2049	77.74	83.3

optimal intakes of ascorbic acid. An increased chloride and water concentration was observed (table 2) in muscles from animals subsisting on a scorbutogenic diet, the magnitude of which appeared to be correlated with the extent of capillary hemorrhage. When due allowance is made for weight changes due to such causes, it is apparent that the rate of regeneration of muscle mass is but little affected by the levels of ascorbic acid intakes. The creatine concentration per unit of dry muscle weight was precisely the same in the scorbutic animals which had subsisted on a vitamin C-free diet for 3 weeks as in the muscles of control animals on the same basic diet supplemented with 10 mg. of ascorbic acid.

DISCUSSION

The gastrocnemius muscles of guinea pigs lose about one-third of their mass and three-fourths of their original strength during the first 2 weeks following denervation. Soon thereafter the first signs of re-innervation appear, atrophy is checked and the affected muscles gradually recover mass and strength. Our findings indicate that the

regenerating muscles of guinea pigs subsisting on ascorbic acid intakes below those considered to be adequate for normal growth rates and protection against scurvy were functionally inferior to the regenerating muscles of animals receiving adequate intakes of the vitamin. This relative weakness of the regenerating muscles was observed for the response to direct as well as to nerve stimulation. In many instances these regenerating muscles exhibited numerous capillary hemorrhages and stood in contrast to the normal appearance of their contralateral controls and to the regenerating muscles of animals supplied with adequate amounts of vitamin C.

Only speculation can be advanced for the presence of hemorrhagic lesions and retarded functional recovery in the regenerating muscles of animals on suboptimal intakes of vitamin C. The crushing of the peripheral nerves deprived the muscles of their sensory as well as their motor innervations and caused a loss of protective reflexes. This resulted in the affected muscles being more vulnerable to stress, strain and trauma. A lack of formation of the proper quality or quantity of collagenous intercellular substances in the supporting tissues may well have contributed to the retarded functional recovery of muscle and nerve from the combined effects of denervation and trauma. The ratios of the tensions developed in response to stimulation of the regenerating nerves to those from direct muscle activation were not significantly different in the animals subsisting on different levels of ascorbic acid intakes. This finding suggests that the relative weakness of the muscles of animals subsisting on the lower levels of vitamin C was not due to ineffective reinnervation but rather to conditions inherent in the muscles themselves. In view of the fact that the lesions of scurvy are much slower to appear in fully grown animals than in growing animals (Wolbach and Bessey, '42), it is possible that the minimal vitamin C requirements are higher for regenerating muscle than for non-regenerating tissue.

Intakes of ascorbic acid considerably in excess of those recognized to be adequate for normal growth and protection against scurvy had no beneficial effect upon the course of neuromuscular regeneration. Our findings indicate that the optimum intake of vitamin C for promoting recovery from peripheral nerve injury is not appreciably different from that recognized to be adequate for the organism as a whole.

SUMMARY

A comparative study was made of neuromuscular regeneration in guinea pigs subsisting on different levels of vitamin C. The ascorbic

acid intakes ranged from 0.5 to 50.0 mg. per day. Complete denervation of the gastrocnemius muscle was produced by crushing the tibial nerve. The contralateral non-denervated limb served as a control. Studies were made at 35 days after operation concerning muscle atrophy and strength and the capacity of the regenerating nerve to activate its muscle.

The regenerating muscles of animals subsisting on suboptimal intakes of vitamin C were relatively weaker than those of animals supplied with adequate intakes. It is suggested that the retarded functional recovery in such muscles may be due to a lack of sufficient collagenous material for regeneration and protection against hemorrhagic lesions precipitated by stress, strain and trauma. The possibility also exists that regenerating muscles may have a higher vitamin C requirement than non-regenerating control muscles. It was found that excess intakes of vitamin C had no beneficial effect upon the course of neuromuscular regeneration.

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RESPONSE TO THE INTRAVENOUS INJECTION OF ASCORBIC ACID AS INDICATED BY THE URINARY EXCRETION OF THE TOTAL AND REDUCED FORMS

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The response to a load or saturation test for ascorbic acid is usually reported in terms of the reduced form of the vitamin. Actually, however, there is no evidence of which the authors are aware, to indicate that the quantitative excretion of the dehydro form of the vitamin might be so negligible as to be unimportant. Although our preliminary work showed that in fasting basal urine samples a rather constant amount (10–20%) of the total excretion of ascorbic acid existed in the dehydro form, there remained the possibility that the same relative results might not obtain following the intravenous injection of 200 mg. of ascorbic acid. The proper interpretation of the response to this load test depends upon a knowledge of the total excretion of ascorbic acid, i. e., reduced plus dehydro forms.

The recent publication of Roe and Kuether ('43) describes a method for the estimation of total ascorbic acid. When this is used in conjunction with the determination of reduced ascorbic acid, the dehydro form can be estimated by difference. Consequently, the matter of the relationships between total, reduced and dehydro ascorbic acid excretion following a load test has been investigated as part of a study of the nutritional status of soldiers, and the results are the subject of this report.

METHODS

The subjects were sixty-eight officers and enlisted men ranging in age from 18 to 42 years, in weight from 128 to 236 pounds, and in height from 5 feet 5 inches to 6 feet 2 inches. For 10 days immediately preceding the load test, all personnel had subsisted on Army Field Ration B which furnished an average daily intake of approximately 100 mg. of ascorbic acid (as determined from actual food consumption and chemical analyses of the food). During the 3 weeks preceding that

time, all had subsisted solely on Army Field Ration A (i. e., the rough equivalent of a liberal civilian diet) in one mess hall. During this time the calculated average daily ascorbic acid intake was in excess of 100 mg.

The following load test procedure was adopted to avoid any interference with the routine military activities of the subjects as well as to avoid error attendant on inclusion of the post prandial excretion of the vitamin. The evening meal, from which all foods high in ascorbic acid had been removed, was eaten at about 6 P. M. At midnight the men were awakened, they emptied their bladders completely, and received intravenously 200 mg. of ascorbic acid,¹ which was administered by the attending medical officer. The intravenous route of administration was selected as preferable to the oral in that it obviated variability in intestinal absorption or destruction. At 6 A. M., the men were again awakened, and excreted the 6-hour urine sample into vessels containing a weighed amount of solid metaphosphoric acid sufficient to prepare a final 2% solution upon further dilution of the urine to a convenient volume. The urine samples were at once transported to the laboratory for immediate analysis.

Reduced ascorbic acid was determined by the photoelectric, 2, 6-dichlorophenol indophenol method of Evelyn, Malloy and Rosen ('38), with a Coleman spectrophotometer. Total ascorbic acid was determined by the colorimetric method of Roe and Kuether ('43) with a Hellige photoelectric colorimeter. In our experience, concentrations of ascorbic acid less than 15 μ g. per milliliter of urine did not produce enough color for accurate estimation by this technique. For this reason, excessive dilution of the urine was avoided, but an appreciable urine volume (200 ml. for the 6-hour period) was encouraged by the administration of a full glass of water at the start of the collection period, whenever the previous day had been hot. It was believed that urine volume of this size would, in addition, serve as a further precaution against possible errors in sampling and from failure to empty the bladder completely. The rare cases of gross deficiency in bladder emptying were detectable by the routine determination of creatinine, which was carried out by a photometric modification of the colorimetric methods of Folin and Wu ('19) and Peters ('42).

RESULTS AND DISCUSSION

A wide variation was noted among individuals in their response to the load test, despite a fairly equal and constant intake of ascorbic

¹Injectable "Cenolate" Abbott ampules testing 104% of labelled strength were used; 4 ml. of the solution being taken up in a sterile syringe with 4 ml. of normal sterile saline.

acid. This was apparent in the estimation of both reduced and total ascorbic acid excretions (table 1). There is a very high correlation ($r=0.914$) between the two methods, indicating a regular association of high reduced ascorbic acid excretion with high total ascorbic acid

TABLE 1

Excretion of total, reduced and dehydro ascorbic acid following intravenous injection of 200 milligrams of ascorbic acid.

SUBJECT NO.	TOTAL ASCORBIC ACID ¹	REDUCED ASCORBIC ACID ²	DEHYDRO ASCORBIC ACID ³	SUBJECT NO.	TOTAL ASCORBIC ACID ¹	REDUCED ASCORBIC ACID ²	DEHYDRO ASCORBIC ACID ³
	mg./6 hrs.	mg./6 hrs.	mg./6 hrs.		mg./6 hrs.	mg./6 hrs.	mg./6 hrs.
1	6.3	3.0	3.3	36	75.0	70.0	5.0
2	48.3	58.5	—10.2	37	8.5	7.6	0.9
3	75.0	83.7	— 8.7	38	30.0	29.2	0.8
4	37.6	33.4	4.2	39	13.8	12.7	1.1
5	71.3	19.9	51.4	40	45.0	43.0	2.0
6	12.0	10.6	1.4	41	8.5	9.4	— 0.9
7	20.0	17.0	3.0	42	31.5	29.8	1.7
8	6.0	4.2	1.8	43	4.3	10.2	— 5.9
9	7.5	5.1	2.4	44	6.0	6.0	0
10	38.8	35.4	3.4	45	20.0	23.0	— 3.0
11	47.5	43.0	4.5	46	69.3	61.5	7.8
12	6.8	5.1	1.7	47	8.3	8.7	— 0.4
13	49.8	48.5	1.3	48	14.8	13.4	1.4
15	9.2	6.8	2.4	49	4.5	7.2	— 2.7
16	9.0	7.6	1.4	50	21.5	18.3	3.2
17	17.8	17.0	0.8	51	7.0	7.0	0
18	10.2	5.2	5.0	52	18.0	14.0	4.0
19	6.4	3.4	3.0	56	4.9	12.7	— 7.8
20	5.6	0.7	4.9	57	46.5	43.3	3.2
21	5.5	4.7	0.8	58	66.8	61.5	5.3
22	6.5	4.3	2.2	59	8.5	11.3	— 2.8
23	4.5	4.7	— 0.2	60	46.3	40.2	6.1
24	7.3	7.6	— 0.3	61	66.5	63.0	3.5
25	5.4	7.2	— 1.8	62	9.0	5.2	3.8
26	32.0	22.8	9.2	63	19.0	13.0	6.0
27	7.5	3.9	3.6	65	42.0	7.9	34.1
28	99.6	63.1	36.5	71	77.6	24.0	53.6
29	80.7	63.6	17.1	72	60.0	53.0	7.0
30	31.5	23.8	7.7	73	60.9	48.6	12.3
31	64.1	59.2	4.9	74	95.0	63.6	31.4
32	6.5	5.2	1.3	75	8.0	0	8.0
33	30.5	27.8	2.7	76	12.5	2.6	9.9
34	8.5	9.8	— 1.3	77	5.3	0	5.3
35	6.3	9.8	— 3.5	78	103.5	102.0	1.5
Means					29.4	24.2	5.2 ⁴

¹ Method of Roe and Kuether ('43).

² Method of Evelyn, Malloy and Rosen ('38).

³ By difference, total—reduced.

⁴ Highly significant mean difference.

excretion. It can be concluded, therefore, that either method would prove equally reliable in determining response to the load test. If it should become apparent that no additional information is provided by the estimation of total ascorbic acid, the choice of method then becomes an individual matter. In our own experience, we have found the estimation of reduced ascorbic acid to be less time-consuming and more suitable for large numbers of urine analyses.

With few exceptions, the dehydro ascorbic acid (determined by difference), was small and rather constant from one man to another, representing, on the average, 18% of the total ascorbic acid excretion.

The difference between the means of the two methods, although small, is highly significant statistically as shown in the analysis of variance table (table 2). This is interpreted as indicating that a small

TABLE 2
Analysis of variance.

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Total	135	91,396	
Between men	67	86,093	1,285
Between methods	1	907	907
Error	67	4,397	66

but nevertheless significant quantity of dehydro ascorbic acid is actually present in addition to the reduced ascorbic acid measured in the total ascorbic acid method. An inspection of table 1 reveals six erratic dehydro ascorbic acid values, i. e., subjects 5, 28, 29, 65, 71 and 74, that might be suspected of being responsible for the significance of the difference between the means of the two methods. When these values are eliminated, however, the difference between the means, although reduced, is still highly significant statistically.

SUMMARY

Reduced and total ascorbic acid urinary excretions were determined on sixty-eight soldiers during a 6-hour period following intravenous injection of 200 mg. of ascorbic acid. Dehydro ascorbic acid has been computed by difference.

The excretion of total ascorbic acid paralleled the excretion of reduced ascorbic acid (correlation $r=0.914$). It is concluded that the measurement of either substance would prove suitable to determine relative load test response.

The means of the total ascorbic acid and the reduced ascorbic acid excretion were 29.4 and 24.2 mg. respectively. The difference between the means of the two methods, although small, is highly significant statistically. This is interpreted as indicating the presence of a small quantity of dehydro ascorbic acid in the urine.

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A SURVEY OF THE ASCORBIC ACID STATUS OF COLLEGE STUDENTS

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ONE FIGURE

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Blood plasma ascorbic acid values of college students in four northwestern universities have been investigated by Brown and others ('43). A similar study was begun in 1941 at the University of Tennessee. The plan was to supplement and generalize the findings of the northwest group by sampling a population differing in locality and food habits.

EXPERIMENTAL

Subjects were college women enrolled in nutrition and physiological chemistry classes open to freshmen, juniors, and seniors. For the determination of the ascorbic acid content of the blood plasma the micromethod of Mindlin and Butler ('38) was used with the Bessey method ('38) of correcting for turbidity. The suitability of this method for survey studies has been verified by Beebe ('42). Blood samples were taken on two non-consecutive mornings after a breakfast containing no ascorbic acid, and all data reported are average plasma ascorbic acid values. A routine record was made of the previous day's dietary and the ascorbic acid content estimated.

RESULTS

A total of 345 students served as subjects of whom 196 were freshmen and 149 juniors and seniors who showed similar findings. The study extended from the spring of 1941 through the spring of 1943. Ascorbic acid values of the two groups were arranged according to the classification of Neuweiler ('39), (table 1). Its use does not imply agreement with Neuweiler's interpretation of the significance of these groupings but does simplify comparison of these findings with those of the northwestern survey which have been so classified.

The distribution of these data showed that approximately 60% of both groups lay within the middle two intervals. Almost 25% of the

freshmen and only 10% of the upper-classmen had plasma levels below 0.4 mg. %. The upper-class group had one-third of their number in the high level above 1.0 mg. %, the freshmen only one-sixth.

The means of the two groups, 0.67 and 0.84 mg. %, as well as the distribution of values indicated a difference in the two groups. Whether such differences of means could exist and still represent a homogeneous population was checked by Fisher's *t* test. The *t* value found, 4.85, exceeded the *t* value, 2.59, given for the 1% level for this size sampling. It may be concluded, therefore, that the two groups represented populations which were different with respect to their ascorbic acid status as shown by blood plasma values and that the upper-classmen had a

TABLE 1

Distribution of ascorbic acid plasma levels for Tennessee women compared with totals for women in the Northwest.

PLASMA ASCORBIC ACID	FRESHMEN		UPPER-CLASSMEN		NORTHWEST TOTALS FOR WOMEN	
	No.	%	No.	%	No.	%
mg./100 ml.						
Less than 0.4	47	24.0	14	9.4	36	7.6
0.4-0.79	82	41.8	56	37.6	199	42.3
0.8-0.99	33	16.8	29	19.5	124	26.3
Over 1.0	34	17.3	50	33.6	112	23.8
Mean plasma ascorbic acid, mg./100 ml.	0.67		0.84		0.797	
Standard error	± .024		± .026		± .0126	

significantly higher level. The mean values and the distribution of these data are similar to those found by the northwest workers, the freshmen tending to parallel their data for men, the upper-class group their data for women.

The results obtained by calculating unweighed food intakes are open to considerable doubt. Approximately 60% of these subjects ate at the university cafeteria and consequently standardization of servings could be assumed. The research students who recorded and calculated the dietaries were familiar with methods of preparation and were competent to weigh values in estimating vitamin content. No claim is made that the values given are more than fair estimates. Dietary intake was based on an average of the days immediately preceding the two blood samplings. There seemed to be little agreement between intake and the following day's plasma value. Averaging the two sam-

plings tended to improve agreement. The average intakes were close together, for freshmen 86.3 ± 3.14 mg. and for upper-classmen 82.9 ± 2.65 mg. There was no significant difference between these two intakes and from this portion of the data it appeared that the two groups had a different ascorbic acid status as shown by the plasma values on identical intakes.

It has been generally assumed that there is close correlation between dietary intake of ascorbic acid and plasma levels. In this study the correlation coefficient, r , was 0.376 for freshmen and 0.398 for upper-classmen. These indicate that the effect of intake in fixing plasma values was only 40%, if a straight line relationship is assumed. An

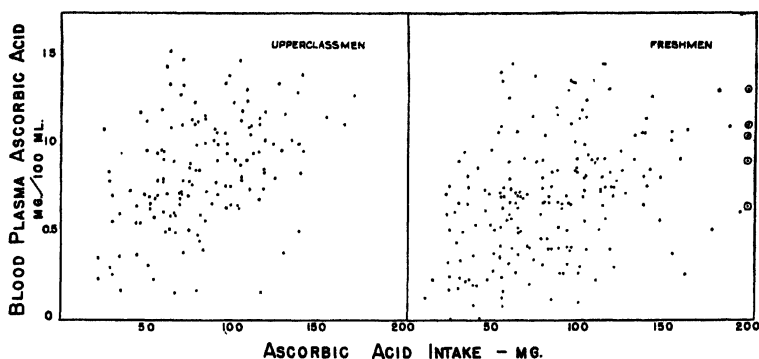


Fig. 1 Ascorbic acid intake and blood plasma values. Circle indicates intake of 200 mg. or above.

r value of 0.51 has been reported by Smith et al. ('42) for 300 subjects in a similar survey in which intake was calculated for 1 week before taking the blood sample. This longer period can be assumed to furnish a more representative estimate of ascorbic acid intake yet the correlation for the two factors was not greatly altered. A carefully controlled intake would be necessary to determine accurately the degree of this dependence, but the present data suggest the existence of other factors which affect a direct relationship of ascorbic acid intake to plasma values. Using Fisher's t test as a criterion, such correlation as exists is highly significant and cannot possibly be due to chance. Scatter diagrams are given for the two groups (fig. 1).

SUMMARY

1. Blood plasma ascorbic acid values obtained by a survey of 345 college women showed the freshmen to have a mean value of 0.67

mg. % which was significantly lower than the mean value of 0.84 mg. % of the upper-classmen.

2. The values obtained in this survey agree well both in means and distribution with findings of other workers using comparable methods of analysis for similar populations differing in locality.

3. Correlation based on a linear relationship of estimated dietary intake of ascorbic acid to blood plasma levels was limited and suggested that (a) such procedure in estimating dietaries is unsatisfactory and/or (b) other factors operate in this relationship.

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THE MAINTENANCE OF ADULT RATS ON DIETS LOW IN CERTAIN B VITAMINS¹

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It appears to be generally accepted that a moderate excess of the essential dietary factors will promote better health and resistance to disease than a diet deficient in any one of them. However, Rasmussen ('43) has observed that mice deficient in thiamine were more resistant to the Lansing strain of poliomyelitis virus than those receiving adequate amounts of this vitamin. Rats on a diet deficient in the vitamin B complex are reported to be less susceptible to chronic ulcerative cecitis (Bloomfield and Lew, '43) or to infection by trypanosomes (Reiner and Paton, '32) than those on a more adequate diet.

Du Vigneaud and coworkers ('42) reported an increased incidence of hepatic tumors due to p-dimethylaminoazobenzene when biotin was added to the basal ration. Miner et al. ('43) observed that adult rats fed the dye and 25 μ g. of pyridoxine per day developed more hepatic tumors than similar animals fed only 2 μ g. of pyridoxine per day. Diets low in pyridoxine tended to diminish both the number of tumor "takes" and the growth rates of the inoculated tumors in rats and mice (Bischoff, Ingraham, and Rupp, '43; Kline et al., '43). Spontaneous mammary carcinomas in C₃H mice were found to grow more slowly when the host animal was fed a diet low in pantothenic acid (Morris and Lippincott, '41), in lysine (Voegtlin and Thompson, '36); Voegtlin and Mavor, '36) or in cystine and methionine (Morris and Voegtlin, '40) than when adequate diets were fed.

Apparently, therefore, pathological conditions exist in which a mild, tolerable dietary deficiency may be preferable to the margins of safety usually prescribed. It is possible, for instance, that levels of nutrients may be found that are lower than the minimum needed for the growth of a virus or tumor, and yet are adequate for a reasonable degree of maintenance of the adult. However, information on the latter requirement is still too meager for an effective evaluation of the possibilities.

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To this end data have been assembled on the health and survival of adult rats fed synthetic diets low in each of the essential B vitamins.

METHOD

Groups of weanling albino rats of the Sprague Dawley strain were placed in screen cages and allowed to grow for 8 to 10 weeks on synthetic diets. They were then placed in single cages and fed "maintenance" diets deficient in individual vitamins of the B complex for a year or until death. Regardless of the condition of the animals this latter period was designated as the "maintenance" period. Food and water were given ad libitum, but the food consumption of each rat was measured bimonthly. The rats were weighed and examined weekly.

TABLE 1

Micrograms of the B vitamins fed per rat per day during the period of growth.

VITAMIN	DIET		
	Low vitamin	Medium vitamin	High vitamin
Nicotinic acid	10	30	60
Thiamine chloride	10	30	60
Riboflavin	15	45	90
Calcium pantothenate	44	132	264
Pyridoxine hydrochloride	10	30	60
Choline chloride	10000	10000	10000

The basal ration for both growth and maintenance contained purified casein,² 18; corn oil, 5; Wesson salts, 4; and glucose,³ 73; plus various amounts of crystalline thiamine chloride, nicotinic acid, calcium pantothenate, riboflavin, pyridoxine hydrochloride, and choline chloride. These vitamins were mixed with glucose before being added to the other ingredients of the diet. The daily allotment of vitamins was contained in 6 gm. of the growth ration until the rats consumed 10 or more grams of food per day; thereafter, and during the maintenance period, this quantity of vitamins was added to 10 gm. of ration. The diets were mixed in 2- to 5-kg. lots and stored at -5°C . Each rat received 1 drop of halibut liver oil per week.

The diets fed during the growth period contained either low, medium, or high amounts of the B vitamins in a ratio of 1 to 3 to 6 times the lowest level (table 1). The medium vitamin diet was adopted as the

² Crude casein was washed for 1 week with several changes of tap water and then extracted with two changes of ethyl alcohol for 4 days at 50°C .

³ Cerelese, a pure commercial glucose monohydrate.

control ration for the maintenance period, except that only 0.3 mg. of choline was contained in 10 gm. of ration instead of the 10 mg. fed during growth. The other maintenance diets differed from this ration in that one or more of the crystalline vitamins were omitted entirely. Such rations were designated as "free" from the factors in question. However, by the methods of analysis indicated each gram of casein contained 0.6 μ g. of thiamine chloride (thiochrome), 0.6 μ g. of riboflavin (fluorometric), < 1 μ g. of pantothenic acid⁴ (growth of *Lactobacillus casei*), and 0.2 μ g. of pyridoxine hydrochloride (growth of *Saccharomyces carlsbergensis*). The glucose used was a highly purified product prepared by hydrolysis; it contained less than 1 μ g. of pyridoxine per gram as determined by the growth of yeast. Apparently, therefore, no rat received more than 1.1 μ g. of thiamine, 1.1 μ g. of riboflavin, 1.8 μ g. of pantothenic acid, or 0.3 μ g. of pyridoxine per day from the dietary constituents other than the crystalline vitamins.

EXPERIMENTAL

Growth period

During the growth period the rats on the medium and high vitamin diets gained 17 and 18 gm. per rat per week and reached average weights of 179 and 193 gm., respectively; those on the low vitamin diet gained an average of only 12 gm. per rat per week to a final weight of 143 gm. On a similar diet in which 8% of the glucose was replaced by brewers' yeast the rats weighed 242 gm. after 6 weeks (33 gm. per rat per week), although each of the crystalline B vitamins except nicotinic acid was present in lower concentrations in the yeast diet than in the synthetic vitamin diet (Sugiura and Rhoads, '41). The markedly greater rate of growth on the former diet, therefore, was probably due to other dietary essentials in the yeast.

Maintenance period

The changes in weight and survival of the rats on the various maintenance diets are indicated in table 2. The rats on the control diet continued to grow for the first 3 months of the maintenance period and thereafter maintained their weights. Those grown on the low vitamin diet tended to make up for their previous deficiency in vitamins and in size by gaining 133 gm. during the first 3 months of maintenance; those

⁴ This determination was made by Dr. A. L. Neal. Under the conditions used 1 μ g. of pantothenic acid per gram of casein was the smallest amount which could be determined.

grown on the medium level gained one-half as much and those raised on the high level one-fourth as much. In contrast to the animals on the various deficient diets (see below) most of the control animals remained in a good general state of health, although some developed respiratory trouble and died comparatively early in the experiment. After a year

TABLE 2
The maintenance of adult rats on deficient diets.

MAINTENANCE DIET	AVERAGE INITIAL WEIGHT	AVERAGE GAIN IN WEIGHT FROM INITIAL WEIGHT ¹ AT			
		4 mo.	6 mo.	8 mo.	12 mo.
	gm.	gm.	gm.	gm.	gm.
Series I: Grown on the high vitamin diet.					
Control	189 (5)	35 (4)	40 (4)	40 (3)	31 (3)
Thiamine-free	170 (3)	.. (0)
Riboflavin-free	197 (3)	-22 (3)	-39 (3)	- 9 (2)	.. (0)
Pantothenic acid-free	200 (6)	12 (5)	18 (3)	.. (0)
Pyridoxine-free	200 (5)	26 (5)	14 (5)	16 (5)	- 1 (5)
Series II: Grown on the medium vitamin diet.					
Control	175 (6)	64 (6)	63 (3)	65 (3)	95 (3)
Nicotinic acid- and choline-free	182 (7)	58 (7)	50 (6)	90 (3)	131 (3)
Thiamine-free	193 (3)	.. (0)
Riboflavin-free	192 (3)	14 (3)	- 8 (3)	- 5 (3)	-68 (1)
Pantothenic acid-free	175 (8)	31 (7)	1 (3)	- 2 (3)	.. (0)
Pyridoxine-free	174 (8)	28 (6)	2 (5)	- 2 (2)	.. (0)
Series III: Grown on the low vitamin diet.					
Control	141 (3)	133 (3)	152 (3)	207 (2)	185 (2)
Nicotinic acid- and choline-free	134 (4)	108 (3)	118 (3)	127 (3)	92 (3)
Thiamine-free	164 (3)	.. (0)
Riboflavin-free	158 (2)	- 6 (2)	-14 (2)	.. (0)	..
Pantothenic acid-free	134 (5)	33 (5)	26 (2)	15 (1)	21 (1)
Pyridoxine-free	134 (5)	59 (5)	55 (4)	58 (3)	62 (1)

¹ Numbers in parentheses indicate the number of animals alive at each period.

of maintenance half of the males weighed 330 gm. and over, while half of the females exceeded 230 gm. in weight.

The omission of both nicotinic acid and choline from the maintenance ration failed to affect the performance of the adult rats. They continued to gain in weight slowly for about 3 months, and most of them still appeared healthy after a year. The deaths that occurred in this group were usually due to respiratory infections. Since the rat appears to be able to synthesize enough of both nicotinic acid (Dann, '41) and

choline (Jacobi, Baumann, and Meek, '41) for a fair rate of growth, it is not surprising that the synthesis of these factors should also be adequate for the maintenance of the adult. In fact for the purpose of the present experiment the rats maintained in the absence of dietary nicotinic acid and choline could be regarded as additional control animals.

In contrast the adult rats deprived of thiamine failed rapidly. Those grown on the diets of low or medium vitamin content gained in weight for only a week after being placed on the maintenance ration free of thiamine. Thereafter they declined rapidly to death in 2 to 4 weeks. When placed on the deficient ration, rats grown on the diet high in thiamine gained in weight for about 2 weeks and survived for a total of 6 to 7 weeks. During the last 2 or 3 weeks of life the rats consumed only 1 to 4 gm. of food per day; they became badly emaciated and many showed poor coordination.

The performance of the rats on the diets deficient in riboflavin, pantothenic acid, or pyridoxine was intermediate between that of the control animals and those deprived of thiamine. Significantly, many of the animals on each of these diets maintained their weights and remained free of gross deficiency symptoms for long periods of time. The rats on the diet free of riboflavin survived for 7 to 12 months. During the first 4 months these rats appeared nearly normal except for a slight loss in weight and lack of activity. Thereafter an alopecia gradually developed until, in some cases, most of the hair on the head, legs, and abdomen was gone. At the same time a dermatitis developed and became progressively more severe. Before death seven of the eight rats had open sores on the neck and paws and occasionally on other parts of the body.

The adult rats fed the diet free of pantothenic acid grew for about 1 month and thereafter either maintained their weights or declined very slowly. The animals remained approximately normal in appearance for 2 to 3 months, after which the chief gross symptoms were a listlessness and lack of muscle tone. "Bloody" noses, whiskers, and forelegs developed in about 70% of the animals before death. Survival was good up to 4 months, but about 50% of the animals died during the fifth and sixth months. Severe losses in weight were observed only during the last 10 to 14 days of life, and on autopsy numerous hemorrhagic areas were found in the intestinal tract, especially in the ileum. No macroscopic hemorrhages were found in the adrenals. Essentially the same results were obtained whether the rats had been grown on diets containing low, medium, or high amounts of the B vitamins.

This conclusion was also drawn from an experiment in which two other groups of weanling rats were grown on the diet free of pantothenic acid plus daily oral supplements of the vitamin in water. One group received 264 μ g. of calcium pantothenate daily by dropper throughout the period of growth, the other 264 μ g. daily during the first week with the subsequent doses decreased each week by 44 μ g. per day. During the eighth week the daily dose was 22 μ g. The rats on the graded dosage grew nearly as well as those fed the high level throughout (the weights after 8 weeks averaged 177 gm. and 206 gm., respectively), and when placed on the maintenance ration free of pantothenic acid, they survived 8 months as compared to 6 months for those receiving the larger doses of the vitamin. Unna and Richards ('42) observed that the requirement of the rat for pantothenic acid decreases with age.

By way of contrast, the maintenance of adult rats on the diet free of pyridoxine appeared to depend upon the amount of pyridoxine consumed during growth. Rats grown to maturity on the higher level of the B vitamins gained in weight during the first 2 months of maintenance and thereafter virtually maintained their weights for another 8 months on the deficient diet. They remained nearly normal in appearance for about 5 months when a loss in muscle tone became the most conspicuous symptom of deficiency. After 12 months on the deficient diet the survival of the animals was still 100%. However, of the rats grown on the low and medium diets 50% died within 9 and 7 months, respectively. In these two latter groups the rats gained in weight for about 1 month, lacked muscle tone after 3 to 4 months, and became hypersensitive in 5 to 6 months. One rat was observed in convulsions after 7 months.

Rats which had consumed only 0.2–0.3 μ g. of pyridoxine daily for 2 months or more excreted 0.1–0.4 μ g. of pyridoxine daily in the urine and 0.5–0.8 μ g. in the feces, as determined by the growth of yeast (Atkin et al., '43). Control rats receiving 36 μ g. of pyridoxine daily excreted about 30 μ g. in the urine; after 1 day on the deficient diet, they excreted 3–8 μ g. during the next 24 hours.

In another series twenty-six weanling rats were grown on the synthetic diet free of pyridoxine plus aqueous supplements of the vitamin orally. One group received 60 μ g. of pyridoxine per day while two other groups received graded doses of the vitamin (table 3). The three groups gained an average of only 14, 10, and 9 gm. per week to average 168, 138, and 130 gm. in weight, respectively, at 9 weeks, as contrasted with an average weight of 193 gm. when 60 μ g. of pyridoxine was incorporated into the daily allotment of the basal ration. The discrepancy

in growth rates was attributed to the rapid excretion of aqueous doses of pyridoxine (Scudi, Buhs, and Hood, '42, Scudi, Unna, and Antopol, '40; Swaminathan, '41).

After 9 weeks of growth the rats were placed either on the maintenance ration free of added pyridoxine, or on a diet containing 2 μ g. of crystalline pyridoxine per 10 gm. of ration, or on the control ration containing 30 μ g. of the vitamin per 10 gm. The maintenance of the rats on the diet deficient in pyridoxine again appeared to depend mainly upon the amount of this vitamin previously consumed. Only

TABLE 3

The effect of pyridoxine consumption during growth on the subsequent maintenance of rats deprived of pyridoxine.

GROWTH PERIOD		MAINTENANCE PERIOD		
Daily oral dose of pyridoxine	Average initial weight	Average gain in weight from initial weight ¹		
		4 mo.	6 mo	8 mo.
μ g.	gm.	gm.	gm.	gm.
No pyridoxine				
60 throughout	171 (4)	0 (4)	5 (4)	— 18 (3)
60, 50, 40, 30, 20 ²	135 (2)	6 (2)	(0)	...
15, 12.5, 20 ²	135 (5)	10 (2)	7 (2)	— 14 (1)
2 μ g. pyridoxine per 10 gm. maintenance diet				
60 throughout	166 (4)	22 (4)	31 (4)	22 (4)
30 μ g. pyridoxine per 10 gm. maintenance diet				
60 throughout	168 (4)	54 (4)	60 (4)	67 (4)
60, 50, 40, 30, 20 ²	143 (2)	72 (2)	87 (2)	97 (2)
15, 12.5, 20 ²	124 (5)	94 (5)	109 (5)	117 (4)

¹ Numbers in parentheses indicate the number of animals alive at each period.

² The first doses were administered for 1 week each; the last dose for the remainder of the growth period. 20 μ g. daily was apparently needed for even slow growth.

two of seven animals grown on the lesser amounts of pyridoxine survived 6 months of the deficiency as contrasted with a survival at 8 months of three out of four of the rats previously fed 60 μ g. of pyridoxine daily. All of the rats fed the ration containing 2 μ g. of pyridoxine per 10 gm. appeared to be normal for at least 8 months.

DISCUSSION

A summary of the maintenance records on the deficient diets (table 4) indicates that an adult rat can be deprived of any one of the B vitamins except thiamine without visible damage for several months. Ultimately, of course, deficiency symptoms develop due to a lack of riboflavin, pantothenic acid, or pyridoxine. If the consumption of pyridoxine has

TABLE 4

Summary: Maintenance of adult rats on diets low in the various B vitamins

MAINTENANCE DIET	PERIOD OF GROWTH	PERIOD SEEMED NORMAL	PERIOD MAINTAINED WEIGHT	PERIOD 50% SURVIVED	DEFICIENCY SYMPTOMS		COMMENTS
					Description	Appeared	
Control	mo. 3	mo. 12	mo. 12	mo. 12	None	mo.
Nicotinic acid- and choline-free	3	12	12	12	None
Thiamine-free	1/2	1/2	1/2	1	Poor co- ordination	1	High levels during growth prolonged life
Riboflavin-free	1	2-3	6	8	Listlessness alopecia dermatitis	3 4 4	
Pantothenic acid-free	1	2-3	4-7	5	Poor tonus "Blood- iness"	3 4	High levels during growth did not prolong life
Pyridoxine-free	1-2	3-5	8-10	8 ¹	Poor tonus Hypersen- sitivity	4 5	High levels during growth markedly prolonged life

¹ Over-all average: Those grown on the high vitamin diet all survived longer than 12 months.

been high during the period of growth, the rat can subsequently survive for at least 12 months on a diet very low in this vitamin. A few preliminary experiments indicate that good survival results when only 2 µg. of pyridoxine per 10 gm. are added to the deficient maintenance diet. In view of the observations cited in the introduction, the present results are sufficiently encouraging to warrant long-term tumor experiments with adult animals fed suboptimal diets.

SUMMARY

1. Rats were raised from weaning to 12 weeks of age on synthetic diets containing 0.1% choline and low, medium, or high amounts of

nicotinic acid, thiamine, riboflavin, pantothenic acid, and pyridoxine. They were then maintained for periods up to a year on a diet containing all of these vitamins or on diets from which one of them had been omitted.

2. On the diet free of thiamine the rats lost weight and died in 3 to 7 weeks. Most of the adults on the other deficient diets survived for many months. Those deprived of nicotinic acid and choline for a year were comparable in appearance, growth, and survival to rats receiving these vitamins.

3. The rats on the diet free of riboflavin lived 7 to 12 months. They soon ceased to grow but appeared normal for about 4 months on the deficient diet, after which characteristic symptoms of deficiency developed.

4. Half of the rats on the diet free of pantothenic acid survived for 5 months. These rats stopped growing in 1 month and lacked muscle tonus in 2 to 3 months. On autopsy hemorrhagic areas were found in the intestinal tract.

5. Rats grown on a diet containing 60 μ g. of pyridoxine per 10 gm. of ration subsequently survived for at least 12 months of maintenance on a diet deficient in this vitamin. Those grown on lesser amounts of pyridoxine lived for an average of 8 months. Growth ceased in 1 month and a general atony developed after 3 months more.

6. It is concluded that adult rats can survive in a reasonably healthy state on very low amounts of certain critical vitamins, and that experiments on the therapeutic value of tolerable nutritional deficiencies are feasible.

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THE B VITAMIN CONTENT OF GROATS AND ROLLED OATS ¹

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A survey of the literature indicates a limited amount of information on the B vitamin content of oats. Schultz et al. ('41) studied the thiamine content of twenty-one samples of oats using the fermentation method and found a range of 4.8–10.3 $\mu\text{g.}$ per gram (.48–1.03 mg. per 100 gm.) and an average of 7.2 $\mu\text{g.}$ per gram. Nordgren and Andrews ('41) used the thiochrome method and reported the average thiamine content of six samples of oats as 4.2 mg. per pound (.94 mg./100 gm.). However, the authors claim that this value is probably higher than the nation wide value for oats since the oats were obtained from a station where the thiamine values of wheat and corn were found to be higher than previously reported values. Moir ('42) in a review article has given thiamine values which are distinctly lower. Snell and Strong ('39) determined the riboflavin content of a few samples of oats, but their values are probably too high since in their microbiological method they did not remove interfering fatty acids. Bacharach ('41) in a review article has given a few nicotinic acid values. In this investigation we have assayed several samples of hulled oats (groats) and rolled oats for their thiamine, nicotinic acid, pantothenic acid, riboflavin and pyridoxine content.

EXPERIMENTAL

Samples of hulled oats were obtained from the three commercial mills ². We also assayed samples of experimental hand hulled oats obtained from Dr. H. L. Shands of the Department of Agronomy. All samples were ground in the Wiley mill with the medium sieve.

Thiamine was determined by the Hennessy-Cerecedo thiochrome fluorescence method as modified by Hennessy ('42). Samples were prepared as follows: One gram of the powdered oats was digested with 75 ml. 0.1 N H_2SO_4 at 100°C. for 1 hour, and then cooled below

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² Cedar Rapids, Iowa; Akron, Ohio; and St. Joseph, Missouri. We are indebted to the Quaker Oats Co. of Chicago, Illinois, for these samples.

50°C. after which 5 ml. of a 2.5 N sodium acetate solution containing 5% clarase were added. It was then kept at 45–50°C. for 1 hour after which it was held overnight in a 37°C. incubator. After filtering, an aliquot portion was adsorbed on a column of Decalco, eluted with 25% KCl in 0.1 N HCl and oxidized with a 0.03% $K_3Fe(CN)_6$ solution in 15% NaOH. The fluorescence of the thiochrome formed was read in the Coleman photofluorometer. Values are expressed as mg. of thiamine hydrochloride per 100 gm.

Pantothenic acid was determined by the microbiological method of Strong et al. ('41). Samples were prepared as follows: 0.5 gm. of powdered sample was suspended in 75 ml. distilled water, autoclaved at 15 pounds pressure for 15 minutes and finally diluted to the necessary volume. Values are expressed as mg. calcium pantothenate per 100 gm.

Riboflavin was determined by the microbiological method of Snell and Strong ('39) as modified by Strong and Carpenter ('42). Samples were prepared as follows: 50 ml. of 0.1 N HCl were added to 5 gm. of powdered sample, autoclaved at 15 pounds pressure for 15 minutes, cooled and then 3.3 ml. of 2.5 molar sodium acetate was added. The mixture was then adjusted to a volume of 100 ml., filtered through Whatman no. 40 filter paper, and 50 ml. aliquot of the clear filtrate adjusted to pH 6.8–7.0 and made up to 100 ml. Trial runs showed that this procedure was adequate for the removal of interfering substances since subsequent extraction with ether gave similar results. Values are expressed as mg. riboflavin per 100 gm.

Nicotinic acid was determined by the microbiological method of Snell and Wright ('41) as modified by Krehl et al. ('43). In this modification higher concentrations of glucose, sodium acetate, and cystine are added to the basal medium. Samples were prepared as follows: 25 ml. of 1 N NaOH were added to 1 gm. of powdered sample, autoclaved at 15 pounds pressure for 15 minutes, neutralized and diluted to the necessary volume. Values are expressed as mg. nicotinic acid per 100 gm.

Pyridoxine was determined by the microbiological method of Atkin et al. ('43). This method employs the response of a strain of yeast to pyridoxine, the response being measured as turbidity readings on the Evelyn colorimeter. Samples were prepared as follows: 1 gm. of powdered sample was suspended in 180 ml. 0.44 N H_2SO_4 , heated at 20 pounds pressure for 1 hour in the autoclave, cooled, neutralized to pH 5.2, and then adjusted to 200 ml. Various levels of the unknown solution were added to 125 ml. Erlenmeyer flasks which contained 5 ml. of the pyridoxine free basal medium and then sufficient distilled

water was added to make a total volume of 9 ml. The flasks were then inoculated with 1 ml. each of the yeast inoculum. They were then kept in a 30°C. incubator for 18 hours where they were shaken two or three times during the period of incubation. This procedure gave consistent results and recoveries. Values are expressed as mg. pyridoxine hydrochloride per 100 gm.

RESULTS AND DISCUSSION

In table 1 are shown the results of analysis of the commercial oats. The table is arranged in pairs giving the B vitamin content of hulled oats (groats) and of the rolled oats prepared from these groats. It

TABLE 1

Vitamin content of groats and rolled oats.

MILL		THIAMINE	NICOTINIC ACID	PYRIDOXINE	PANTOTHENIC ACID	RIBOFLAVIN
		mg./100 gm.	mg./100 gm.	mg./100 gm.	mg./100 gm.	mg./100 gm.
Cedar Rapids, Iowa	Groats 1	0.686	0.96	0.130	1.39	0.127
	Rolled oats 1	0.672	0.90	0.100	1.35	0.120
Cedar Rapids, Iowa	Groats 2	0.612	0.78	0.104	1.58	0.137
	Rolled oats 2	0.582	0.72	0.099	1.47	0.130
Cedar Rapids, Iowa	Groats 3	0.636	0.84	0.104	1.82	0.135
	Rolled oats 3	0.636	0.86	0.104	1.75	0.133
Akron, Ohio	Groats 4	0.734	0.69	0.104	1.26	0.126
	Rolled oats 4	0.672	0.66	0.104	1.14	0.119
Akron, Ohio	Groats 5	0.690	0.82	0.119	1.65	0.150
	Rolled oats 5	0.668	0.78	0.098	1.65	0.141
Akron, Ohio	Groats 6	0.746	0.88	0.116	1.17	0.129
	Rolled oats 6	0.652	0.86	0.122	1.09	0.122
St. Joseph, Missouri	Groats 7	0.800	0.90	0.130	1.75	0.155
	Rolled oats 7	0.784	0.88	0.135	1.67	0.146
St. Joseph, Missouri	Groats 8	0.827	0.96	0.148	1.62	0.167
	Rolled oats 8	0.790	0.96	0.149	1.62	0.159
St. Joseph, Missouri	Groats 9	0.830	0.92	0.122	1.60	0.135
	Rolled oats 9	0.812	0.94	0.120	1.60	0.130

AVERAGE VALUES

Cedar Rapids, Iowa	Groats	0.645	0.86	0.113	1.60	0.133
	Rolled oats	0.630	0.83	0.101	1.52	0.128
Akron, Ohio	Groats	0.723	0.80	0.113	1.36	0.135
	Rolled oats	0.664	0.77	0.108	1.29	0.127
St. Joseph, Missouri	Groats	0.819	0.93	0.133	1.69	0.152
	Rolled oats	0.795	0.93	0.138	1.63	0.145

can be seen that there is only a slight loss in the pyridoxine, thiamine, riboflavin, nicotinic acid and pantothenic acid content in the preparation of rolled oats from groats and in many cases this apparent loss is within the limits of the experimental error.

From table 1, it can also be seen that there is a tendency for the groats and rolled oats from the St. Joseph, Missouri, mill to be higher in all the B vitamins determined. This may be due to variation in oats obtained from different regions.

TABLE 2
Vitamin content of experimentally hand-hulled oats.

VARIETY	THIAMINE	NICOTINIC ACID	PYRIDOXINE	PANTOTHENIC ACID	RIBOFLAVIN
	mg./100 gm	mg./100 gm	mg./100 gm.	mg./100 gm	mg./100 gm.
1. States Pride Ped. 7	1.03	1.35	0.163	1.75	0.137
2. Vieland	1.20	1.10	0.172	2.35	0.144
3. Tama	1.04	1.05	0.139	2.50	0.151
4. Erban	1.21	1.25	0.160	3.50	0.148
5. Marion	1.32	1.20	0.160	2.70	0.143
6. X215-19	1.17	0.725	0.152	2.20	0.126
7. X219-1-2	1.22	1.08	0.142	2.75	0.182
8. X219-1-3	1.13	0.825	0.257	2.40	0.250
9. X220-13	1.23	0.925	0.196	2.45	0.165
10. X254-1-1	1.22	1.15	0.207	2.85	0.155
11. X254-6	1.00	1.50	0.313	2.20	0.220
12. X306-1	1.17	1.22	0.169	3.20	0.196
13. C. I. 3664	1.22	1.00	0.203	2.50	0.145
14. C. I. 4170	1.00	0.95	0.300	1.70	0.133
AVERAGE VALUES	1.15	1.095	0.195	2.50	0.164

In table 2 are summarized the values obtained on the experimentally produced oats. It is evident that these hand hulled oats are distinctly higher in the B vitamins than those summarized in table 1. The values for thiamine show the largest and those for riboflavin the smallest differences. These variations are not surprising since the experimental oats were produced on plots that were well fertilized and were harvested under controlled conditions. The hulls were removed by hand which allowed not only selected samples but may have allowed a greater retention of vitamins. It is also interesting to note that the experimental oats were made up of several varieties and that no one variety was consistently higher than the others in all the vitamins.

While the results presented in this paper give a general picture of the B vitamin content of oats it should be emphasized that samples collected from other areas of the country and grown under other environ-

mental conditions may differ somewhat in composition. The methods used for the estimation of the vitamins were very satisfactory and almost identical results were obtained when the same sample was analyzed at different periods. However, some difficulty was encountered when the thiamine determinations were made on the same original sample but ground under slightly different conditions. This difficulty is apparently encountered only in the case of oats, and until means are found for completely eliminating this variation it may be necessary to allow a 10% differential in the thiamine results.

SUMMARY

The thiamine, nicotinic acid, pantothenic acid, riboflavin and pyridoxine content of about thirty samples of commercial oat groats and rolled oats, and experimentally hand-hulled oats, are given.

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STUDIES OF THE COMPARATIVE NUTRITIVE VALUE OF FATS

II. THE COMPARATIVE COMPOSITION OF RATS FED DIFFERENT DIETS ¹

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In an earlier study from this laboratory, no significant differences were noted in the rate of growth of white rats over a 12-week period following weaning (Deuel, Movitt, Hallman and Mattson, '44) when different fats were added to a basal diet of mineralized skimmed milk powder and vitamin supplements, in an amount of 29.4%. The fats tested were butter and margarine, and corn, cottonseed, olive, peanut, and soybean oils. The present study was designed to determine whether any differences obtained in the composition of the rats which had received the different diets over a period of 12 weeks.

The present tests were made on 102 rats of series II; the details of the experimental procedure are described earlier (Deuel, Movitt, Hallman and Mattson, '44). After 12 weeks on the various diets, the animals were anesthetized with sodium amytal, weighed, the stomach and intestines were removed and weighed, and the carcass was ground to a homogeneous mass by passing through a meat grinder three times. The weight of the rat and the water content of the aliquot were corrected for the amytal injected.

Water was determined by drying aliquot samples to constant weight in the vacuum oven at approximately 60°C. The dried tissue was then extracted for 16 hours in a Soxhlet extractor with diethyl ether. After removal of the ether, the tissue was reground in a Wiley mill and the dried, defatted powder used for determination of nitrogen by the Kjeldahl method, and of ash. Calcium was determined on the ash by

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titration of the oxalate with KMnO_4 as described by Hawk and Bergeim ('37). Carbohydrate was estimated by difference.

The results are summarized in table 1.

The composition of the rats is quite uniform in the different groups but varies with the sex of the animals. In no case was any statistically valid difference found between groups. The values for protein vary in the males between 17.8 and 16.2% and in the females between 17.0 and 15.6%. These are somewhat lower than other values in the literature, i. e., 18.75 (Terroine et al., '23, '24), 18.83 (Light et al., '34), 20.5 (Truszkowski, '26), and 21.1% (Chanutin, '30). The ash content varied in the males between 2.87 and 3.16% and in the females between 3.22 and 3.28%. These values agree well with the 3.38% reported by Light et al. ('34) and the 3.10% obtained by Buckner and Peter ('22) for 16-week-old rats.

On the other hand there is a definite sex difference in the composition of the rats. In general, the males have a higher water and protein content and a lower percentage of lipid, ash and calcium. Although these differences are usually not significant statistically when the comparisons are made for the animals on a single diet, it is to be noted that these variations are found in every average with the various components mentioned above (except in one case with the percentage of water). Based on a P value of 0.01, the "t" values² for the variations in ash and calcium are significantly different between the male and female rats on the corn oil, cottonseed oil, margarine and peanut oil diets. When the statistical evaluation is made on the average analyses of all the males and females, the differences are significant for protein, ash and calcium and practically so for water and lipid. The sex variation in calcium content is in agreement with earlier observations of Lanford, Campbell and Sherman ('41). Although the absolute values varied with the age of the rats, the percentage of calcium of the 90-day-old rats on several diets was as follows: diet 13 (0.34% Ca) males 0.899, females 1.066; diet 132 (0.48% Ca) males 0.968, females 1.126; diet 133 (0.64% Ca) males 1.000; females 1.187. These values are higher than obtained in our tests (males 0.911, females 1.028) where the diet had 0.88% Ca. However, our diets contained approximately 30% of fat whereas in the above tests the diets had one-third skimmed milk powder and two-thirds whole wheat flour, the increased calcium being supplied with the last two diets by the addition of CaCO_3 .

²In all cases the "t" value exceeded that required for a "P" value of 0.01 (one chance in 100 that the results occurred by chance). This method of statistical evaluation is more accurate when the number of cases is under ten (Fisher, '36).

TABLE 1
The average composition of the tissues of eviscerated rats which had previously received a mineralized skimmed milk diet with different fats for 12 weeks after weaning at 3 weeks of age.

SEX	BODY WEIGHT		COMPOSITION ON WET BASIS IN PER CENT ¹					
	Normal	After evisceration	Water	Protein	Lipid	Carbohydrate	Ash	Ca
Male (51 rats)	gm. 254.8	gm. 237.0	58.2±0.4 (59.8—56.8)	17.3±0.2 (17.8—16.2)	18.1±0.5 (20.5—16.2)	3.38±0.37 (4.01—2.64)	2.95±0.03 (3.16—2.82)	mg./% 911±11 (958—891)
Female (51 rats)	171.2	158.3	56.8±0.4 (57.8—54.7)	16.4±0.2 (17.0—15.6)	20.1±0.6 (23.3—19.2)	3.53±0.12 (3.99—3.12)	3.26±0.04 (3.30—3.22)	1028±16 (1060—1004)
M.D.: S.E.M.D. ²			2.45	3.10	2.70	0.40	6.20	6.03

The values in parentheses are the limits of the means of the groups on the various diets.

¹ Including the standard error of the means calculated as follows: $\sqrt{\frac{\epsilon}{n} \frac{d^2}{n}} / \sqrt{n}$

² Mean Difference: Standard Error of Mean Difference. When this exceeds 3, the results are considered significant. The comparisons in each case are between the same components in the male and female rats.

SUMMARY

No differences were found in the composition of rats which had received diets of mineralized skimmed milk powder and vitamin supplements together with butter or margarine fat, corn, cottonseed, olive, peanut or soybean oils over a 12-week period. However, it was noted that male rats have a slightly higher water and protein content and a lower lipid, ash and calcium content than the females.

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STUDIES OF THE COMPARATIVE NUTRITIVE VALUE OF FATS

III. THE EFFECT OF FLAVOR ON FOOD PREFERENCE ¹

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Several factors are involved in the regulation of appetite. Probably foremost of these is the instinctive preference of animals for required nutrients which is exercised even to a limited extent by the young animal (Carlson, '16). The selection of salt solution (Richter, '36) and a low potassium diet (Zwemer and Truszkowski, '36) by adrenalectomized rats as well as the choice of calcium lactate solution (Richter and Eckert, '37) by parathyroidectomized rats are instances of such phenomena. Rats will choose qualitatively and quantitatively the protein diets enabling the best growth (Osborne and Mendel, '18) as well as the ones containing adequate vitamins A and undifferentiated B (Mitchell and Mendel, '21). Richter, Holt and Barelare ('37, '38) found that rats would select a diet adequate for normal growth and reproduction from a number of purified foodstuffs simultaneously and separately available. Although flavor may play an important role in the determination of appetite, it is operative only when the diets are reasonably complete; it is entirely ineffective when deficiencies in the diet exist. Thus, Cowgill, Deuel and Smith ('25) were unable to stimulate the appetite of B-deficient dogs with beef extract although moderate doses of a vitamin B concentrate restored the dog's appetite for the bland synthetic diet.

However, on complete diets, flavor may play a role in food consumption with rats as well as with man. The aversion of rats to diets containing rancid fats is well known; the recent report of Clausen, Barnes and Burr ('43) emphasizes the relationship of rancidity to the

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destruction of the B vitamins, particularly when mixed with the food in pure form. In the experiments of Boutwell et al. ('41) where a comparison is made of the growth of weanling rats on mineralized skimmed milk homogenized with butterfat or with vegetable oils, the greater growth of the rats receiving the butter was associated with a greater consumption of this diet. Although admitting that the greater growth of the butter group was associated with a higher food intake, these authors state that increased appetite with the consequent augmented food consumption is an attribute of a superior food.

In earlier papers (Deuel et al., '43, '44) we have reported growth experiments on a diet containing mineralized skimmed milk powder mixed with butter, margarine, corn, cottonseed, olive, peanut or soybean oil. Although no differences in growth were noted at 3 weeks as reported by Schantz et al. ('40), somewhat greater growth obtained in the butter group at 12 weeks with the females although the differences were not noted in the experiments on male rats. Inasmuch as there was some evidence that the greater growth was the result of a larger food consumption, the present study was undertaken to determine whether rats prefer a diet containing butter to one containing other fats. Secondly, after this preference was demonstrated, it was next important to determine whether the preference for the butter diet was an instinctive one for the fat itself or whether it was related to the components in the butter responsible for its distinctive flavor. Tests were then made on the comparative food intake when rats were offered two identical diets one of which was flavored with diacetyl or a commercial butter flavor and one unflavored. The quantity of diacetyl preferred by the rat was also determined.

METHODS

The experiments were made on 21-day-old rats from our stock colony over periods of 3 to 12 weeks. The mothers were on the stock diet prior to the weaning of the litter. The rats were kept in separate cages and the two foods being tested were offered in identical food cups. When the spilling of the food exceeded the difference in apparent food consumption of the two diets, the experiment was discarded.

In the experiments which were carried out over 12 weeks, another procedure was adopted. Two of the individual cages were connected; both had water bottles, and one diet was placed in one cage and the other diet in the second cage. If any spilling occurred, any food so lost could be weighed and correction made. The position of the food cups was reversed daily.

The diet was the same as that described earlier (Deuel et al., '44). Diacetyl² and butter flavor³ obtained commercially were used.

RESULTS

In the first series of tests, the butter diet and one of the diets containing the other fats (corn, cottonseed, olive, peanut or soybean oils or margarine) were offered simultaneously. These results are summarized in table 1.

In every series of tests the butter diet was consumed in the larger amount. The maximum difference was found in the case of the animals receiving also the soybean oil diet where the butter diet was taken in a proportion of more than two to one. The preference for the butter diet is also evident in the number of weeks where a greater amount was consumed. On this basis the highest choice for the butter diet was evident when margarine was also offered, the butter diet being preferred in a proportion of over 6 to 1 compared with the margarine diet.

That the preference for the butter diet is related to its flavor, is indicated by the tests recorded in table 2. This gives the comparison in food intake and diet preference on unflavored peanut or margarine diets and on similar diets flavored with diacetyl or with commercial butter flavor. The level of the flavor preferred is also shown.

DISCUSSION

The experiments reported in table 1 demonstrate that in the majority of instances rats prefer a complete diet where the fat is butter to one where this fat is replaced by vegetable fats. The total score was 106 cases where the butter diet was consumed in greater quantity to 55 where the vegetable fats were preferred. When compared with each fat, the butter was preferred. The results range from a practically even choice with olive oil (13 to 12) and cottonseed oil (15 to 13) to a more marked preference with corn oil (17 to 11) and a decided variation with the other three fats (peanut oil 18 to 10, soybean oil 23 to 6, and margarine 20 to 3). These differences are more clearly evident, however, on the basis of food consumption where in every case the amount of butter diet ingested is definitely greater than that of the vegetable fat. These vary from an amount of 24.3 to 20.6 gm. per rat per week with the butter and corn oil diets respectively to values of more than 2 to 1 in favor of the butter with the butter and soybean oil groups (29.0 gm. to 13.5 gm.).

² Prepared by Larkin and Co., Buffalo, N. Y.

³ Verley B F A obtained from Verley Products Corporation, 1621 Carroll Ave., Chicago, Ill.

TABLE 1

The average food consumption of rats and their dietary preference when a butter diet (B) and one containing a vegetable fat (A) were offered simultaneously over a 3-week period after weaning.

DIET COMPARED WITH BUTTER DIET	AVERAGE FOOD CONSUMPTION PER RAT PER WEEK										DIET PREFERENCE					
	1st week		2nd week		3rd week		AVERAGE		1st week		2nd week		3rd week		TOTAL	
	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
	gm. (9)	gm. (9)	gm. (8)	gm. (8)	gm. (8)	gm. (8)	gm. (25)	gm. (25)								
Corn oil	13.1	13.8	25.7	24.6	35.4	24.2	24.3	20.6	5	4	6	4	6	3	17	11
Cottonseed oil	15.5	13.0	25.4	16.8	30.7	27.4	23.2	18.1	5	5	5	5	5	2	15	13
Margarine	22.8	11.4	26.0	15.6	38.6	23.3	28.5	16.2	8	1	7	1	5	1	20	3
Olive oil	9.0	17.4	21.9	17.6	36.7	19.2	23.7	18.1	2	7	4	4	7	1	13	12
Peanut oil	15.7	11.7	25.5	14.0	29.6	25.3	23.5	17.2	6	4	6	2	6	4	18	10
Soybean oil	19.4	9.5	30.7	12.1	37.3	18.9	29.0	13.5	7	3	8	1	8	2	23	6

The figures in parentheses are the number of experiments upon which the average is based.

TABLE 2

The comparative food intake and preference of rats for diets flavored with diacetyl or commercial butter flavor to similar unflavored diets.

FLAVOR	BASAL DIET	FLAVOR ADDED ¹		NO. OF RATS	WEEKS OF TESTS	TOTAL NO. OF TESTS	FOOD EATEN		DIET PREFERENCE		
		Diet 1	Diet 2				Diet 1	Diet 2	1	2	Tied
Diacetyl	Margarine	p.p.m.	p.p.m.				gm.	gm.			
		0	4	4	2	8	5.0	21.8	0	8	0
		4	8	4	3	11	25.7	14.1	8	3	0
		8	16	4	3	9	24.1	13.3	7	2	0
Peanut	Peanut	16	24	3	3	8	21.4	19.9	3	5	0
		0	4	4	3	12	9.2	34.0	0	12	0
		4	8	4	3	11	16.1	18.5	5	6	0
		8	16	4	3	10	15.6	18.3	6	4	0
Diacetyl (1) and butter flavor (2)	Margarine	16	24	4	3	10	24.9	9.8	8	2	0
		4	4	10	3	30	20.7	27.9	7	22	1
Butter flavor Group I ² Group II	Margarine	0	4	8	12	92	31.5	41.3	19	69	4
				4	12	44	40.8	38.9	21	21	2
Group I ² Group II	Peanut	0	4	8	12	95	27.3	41.3	18	77	0
				4	12	48	34.5	36.7	22	25	1

¹ Parts per million in fat of diet.

² The division into groups is based solely on food preference in an experiment on twelve animals carried on simultaneously.

The preference for the butter diet would seem to be because of the unique flavor which this fat possesses. If this flavor is similar in rat-milk fat to cow-milk fat, the rat would become conditioned to this taste during the period of suckling. The remarkable delicacy of this response is indicated by the fact that it can be brought about by the addition of commercial butter flavors in an amount of 4 parts per million parts of fat or 1.3 parts per million parts of diet. When fed in this concentration with margarine or peanut oil diets, the flavored diets were taken in larger amounts in all twenty tests. The average food consumed of the unflavored and flavored diets leaves no doubt about the preference of the rats (margarine diet, 5.0 to 21.8 and peanut oil diet, 9.2 to 34.0 gm.). With the margarine diet, higher amounts are less acceptable to the rat than the lower concentrations of diacetyl; on the other hand, no preference was noted with the peanut oil diets except at the highest levels where 16 parts per million were decisively preferred to 24 parts per million. Further, it has been shown that the commercial butter flavor in which diacetyl is present to only 12% is a flavor even more acceptable to the rat, being preferred twenty-two times to seven for the diacetyl diet. A laboratory prepared butter flavor containing monobutyryl, butyric acid and diacetyl to simulate commercial butter flavor gave similar results.

The food consumption with the diet having the butter flavor averages 40% higher than in the diacetyl-flavored diet. Also apparently the rats prefer this at the lower level (4 parts per million) than at one which would give 4 parts of diacetyl (33 parts per million).

These differences in food intake are quite marked over an entire 12-week period. In each of twelve experiments carried out on margarine or peanut oil diets flavored with commercial butter flavor or unflavored, eight rats in each group consistently preferred the flavored diet, the total score being 69 to 19 (with 4 tied) in the margarine diet, and 77 to 18 with the peanut oil diet. With respect to the average food consumption of the two types of each diet the same differences are evident, the flavored and unflavored diets being consumed on an average as follows: margarine diet, 41.3 and 31.5 gm. per rat per week; peanut oil diet, 41.3 and 27.3 gm. With four rats in each group no consistent differences in food intake were to be found. The total score was 21 and 21 (with 2 tied) with the margarine tests, and 25 to 22 (with 1 tied) with the peanut oil diets. Also the average food consumption of the flavored and unflavored diets was practically identical in these two series of tests. It is important that in these groups of rats which were

indifferent in their choice of diets, in no case was there evidence of preference for unflavored food.

The preference of rats for a butter diet over one containing vegetable fats which Boutwell et al. ('41) and we have demonstrated appears to be related to the flavor, part of which is dependent on its diacetyl content. There is no evidence that diacetyl or butter flavor possesses any properties which would include them in the category of vitamins. Not only may they be manufactured in the adult lactating female, but also as far as known they do not possess any physiological function in the animal body.

SUMMARY

Rats prefer a diet of mineralized, vitamin fortified skimmed milk powder containing butter to one where the fat is corn, cottonseed, olive, peanut or soybean oil, or margarine. The preference is apparently associated with flavor. The animals universally preferred a margarine or peanut oil diet containing 4 parts per million of diacetyl to an unflavored diet, also, this low level in general is preferred to 8, 16 or 24 parts per million of diacetyl. Commercial butter flavor is favored over diacetyl. It is concluded that flavor may play an important role in determining food consumption of diets which are satisfactory from a nutritional standpoint. Although this would not be consistently demonstrated in all animals with flavored and unflavored margarine or peanut oil diets, two-thirds of the rats consistently chose in a ratio of 4 to 1 the flavored diet while no preference was evident in the remaining animals between the two diets. In no case was there a consistent preference for the unflavored diet.

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STUDIES ON THE URINARY EXCRETION OF RIBOFLAVIN ¹

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ONE FIGURE

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It is generally agreed that riboflavin is an essential constituent of the human dietary but there is not such general agreement concerning the amount necessary and the symptoms produced by a deficiency. In view of this an objective laboratory test which will give an index to the status of the individual with respect to riboflavin becomes of considerable importance. Since riboflavin is water soluble it might be expected that it would appear in the urine and that the amount in this fluid might give an index to the supply available in the body. Three other factors also point in this direction: (1) the urine contains only free riboflavin, (2) riboflavin has the property of fluorescence, and (3) the compound and therefore the fluorescence are readily destroyed quantitatively by exposure to light. The latter two facts can be made the basis for a simple and rapid method of determination.

Several investigators have in consequence determined the urine content of riboflavin (Ferrebee, '40; Williams et al., '43; Swaminathan, '42; Strong et al., '41; Helmer, '37; Emmerie, '36; Sebrell et al., '41; Axelrod et al., '41; Najjar and Holt, '41; Najjar, '41). Four conclusions can be drawn from the results presented in these papers: (1) the 24-hour excretion of riboflavin in the urine varies rather widely among normal individuals on a presumably normal diet, (2) only a fraction of that ingested is excreted, (3) increased intake leads to increased excretion and vice versa, and (4) figures of the same order of magnitude are obtained in widely scattered parts of the world.

The variability in the urinary output has led to the conclusion (Gyorgy, '42) that determinations of riboflavin in the urine are of little if any value in detecting incipient or long standing deficiency. However, in view of the ease with which the determinations can be carried out, it seemed to us that before the idea was discarded further

¹ Aided by a grant from the Nutrition Foundation, Inc.

attempts should be made to test the adequacy of laboratory determinations as an index of riboflavin status.

METHODS

We have used the method and in general the procedure of Najjar ('41). The direct method has been used in all cases and has proven quite satisfactory in our hands.² When two extractions are carried out, this method allows a reading after the destruction of the riboflavin by light, and therefore, a blank reading on each sample. Complete disappearance of the riboflavin was checked for each batch of urine extracts by carrying a standard amount of riboflavin through the procedure including the exposure of an aliquot to sunlight. To date we have carried out such determinations on 658 urine specimens. The samples have been obtained from several widely separated groups and in collecting them we have had two ends in view: (1) to ascertain whether the determination of the urinary riboflavin provides reliable information as to the status of the individual for this vitamin, and (2) to determine whether the average person in this locality has adequate amounts of riboflavin in his diet.

Determinations have been carried out on samples from laboratory workers, hospital and private patients, medical students, men called by a local draft board for physical examination, and a large group of individuals of various ages and economic status from a rural area of Fulton County. In some instances 24-hour urine samples were obtained but usually only fasting morning specimens were analyzed as recommended by Najjar. Where it was not possible to obtain morning samples, hourly specimens were collected and an attempt made to ascertain whether the most recent meal contained substances high in riboflavin such as milk and liver.

RESULTS

The results obtained are shown in figure 1. When these figures were collected the question arose as to how they should be expressed. The 24-hour excretion value seems to have only limited significance (Najjar and Holt, '41) and the alternatives were to calculate either the hourly excretion or the amount per milliliter of urine. When our results were examined, there seemed for the most part to be better correlation between the riboflavin found per unit of volume than per unit of time. Table 1 serves to illustrate the type of result obtained when both

² Readings were made on a Pfaltz and Bauer, Model B, Fluorophotometer.

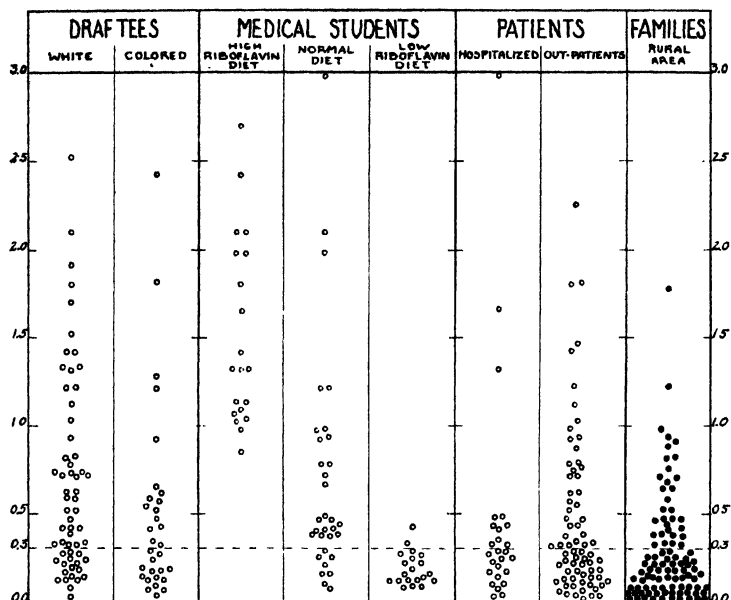


Fig. 1 Riboflavin excretion. Solid circles represent fasting morning samples while open circles represent casual hourly samples.

methods of calculation are applied to the values from four subjects selected from our series. It will be noted that the hourly urine volumes vary widely, in one case (M.M.) nearly ten-fold and the riboflavin excretion in micrograms per hour falls within about the same limits; however, when the output of riboflavin is expressed in micrograms per milliliter of urine, the values for the two urine samples are identical. It is also of interest that the urine specimens in this instance were collected some 9 weeks apart.

From our data, of which table 1 gives only a sample, we became convinced that the excretion per milliliter of urine is the more constant

TABLE 1

*Comparison of fasting riboflavin excretion per hour and per milliliter.
Illustrative data.*

SUBJECT	S.G.		M.M.		M.W.		I.N.	
Date	8/4	8/5	8/25	12/1	9/22	9/29	8/17	8/22
Urine for hr., ml.	45	130	24	208	202	44	207	94
Riboflavin, $\mu\text{g./hr.}$	15.3	43.2	1.4	12.5	114.4	25.0	26.3	14.1
Riboflavin, $\mu\text{g./ml.}$	0.34	0.33	0.06	0.06	0.56	0.57	0.12	0.15

figure and in subsequent work we have used this method of expressing our results.

Our next concern was in establishing a level for urinary riboflavin which should represent a division between the excretion resulting from an adequate intake and that resulting from a diet deficient in this substance. Swaminathan ('42) found that with an intake of 1.2–1.5 mg., 25–30% of the ingested riboflavin appeared in the urine. On a dietary level of 2–3 mg. per day, Sebrell et al. ('41) report that 28–48% was excreted by the kidney. The values of Williams et al. ('43) also fall within this range. A typical individual, then, with a riboflavin intake of 2–3 mg. per day which is the amount advocated by the Committee on Food and Nutrition of the National Research Council, might be expected to have a urinary output in each 24 hours of 500–1000 μ g. with an average of perhaps 800 μ g. For an average urine volume of 1000 ml. this would be 0.8 μ g. per milliliter; for a volume of 1500 ml., 0.53 μ g. When it comes, then, to fixing a value for riboflavin excretion per unit volume above which an individual will be considered adequately supplied with riboflavin and below which he will be considered deficient, it seems that perhaps 0.53–0.8 μ g. per milliliter should be the figure selected. Even though we have for the most part dealt with fasting samples, if the organism is adequately supplied with riboflavin, this level of excretion should persist through the 15 hours of fasting; however, we have felt that for our purposes it would probably be better to set the figure considerably lower and thus avoid the charge of telling an individual he was suffering from a deficiency when in reality he was merely somewhat below the optimum nutrition level. Therefore, for the interpretation of our results we have set 0.3 μ g. per milliliter as the arbitrary level below which a person will be considered deficient in riboflavin. Further support for this may be seen in figure 1 where it is shown that a liberal intake of riboflavin led with a single exception to an excretion of 0.9 μ g. or more per milliliter. A low intake, on the other hand, resulted with a single exception again in an excretion of 0.3 μ g. per milliliter or less.

We have also in several instances carried out analyses on a single morning hourly sample collected separately from that for the remaining 23 hours. For the most part the micrograms of riboflavin per milliliter in the hour sample agreed well with the corresponding figure for the larger sample. This is further justification for considering the micrograms per milliliter of riboflavin in a fasting morning specimen as an index of the body's supply.

There is a further step which has been advocated in assaying the status of an individual with respect to nutrient materials and some attempt has been made to apply it to riboflavin. This is the so-called saturation or load test and we have carried out a number of such tests in connection with the determinations on fasting samples referred to above. The results of these analyses are shown in table 2. We felt

TABLE 2
Riboflavin saturation tests.

SUBJECT NO.	RIBOFLAVIN ADMINISTRATION		RIBOFLAVIN EXCRETION						
	Route	Amount in μ g.	Fasting μ g. per ml.	Percentage of intake during				Total	
				1st hr.	2nd hr.	3rd hr.	4th hr.	Amount in μ g	Per cent of intake
13	I.M. ¹	1000	0.00	13.8	5.7	1.5	0.0	209.9	21.0
19	Orally	1620	0.00	9.45	0.39	11.72	7.07	463.96	28.6
8	I.M.	1000	0.045	6.9	2.3	6.5	3.2	189.1	18.9
13	I.M.	1000	0.10	8.22	1.29	3.45	1.09	140.5	14.05
21	I.M.	1500	0.115	9.23	4.50	2.05	1.30	256.6	17.1
17	I.M.	1500	0.15	0.69	1.32	2.50	4.28	132.01	8.8
8	I.M.	1000	0.18	6.2	6.2	1.8	4.0	181.8	18.2
2	I.M.	1090	0.21	10.0	7.5	3.7	1.7	252.2	22.9
4	Orally	880	0.23	24.0	7.2	5.4	6.11	376.4	42.8
10	I.M.	1000	0.25	27.4	2.1	6.6	4.4	405.4	40.5
15	I.M.	1500	0.26	18.20	3.45	3.93	3.10	430.25	28.7
22	I.M.	1750	0.26	19.3	10.62	6.53	5.20	730.5	41.74
8	I.M.	1000	0.28	6.1	5.08	5.95	0.61	177.44	17.74
5	Orally	768	0.40	37.9	16.1	13.2	10.1	588.7	76.4
21	I.M.	1500	0.406	9.67	6.36	2.87	1.99	313.4	20.9
3	I.M.	1250	0.41	15.12	9.20	12.72	3.72	501.7	40.76
16	I.M.	1000	0.47	17.83	12.4	6.36	6.83	434.2	44.42
3	Orally	1230	0.54	16.0	11.5	6.4	1.9	441.4	35.8
14	I.M.	1000	0.58	17.5	10.1	5.2	2.3	351.9	35.2
19	I.M.	1620	0.59	11.39	9.10	6.12	5.33	517.5	31.9
1	Orally	1250	0.64	21.20	13.15	5.76	3.39	544.4	43.5
9	I.M.	750	0.64	28.2	20.9	13.2	15.0	579.6	77.3
17	I.M.	1500	0.67	6.14	4.76	4.40	0.54	237.67	15.7
6	I.M.	1200	0.76	12.1	14.5	21.8	11.5	719.8	59.9
6	I.M.	1200	0.80	16.7	17.5	7.7	9.1	612.3	51.0
7	I.M.	1340	0.82	22.1	15.7	7.8	7.6	715.3	53.4
9	I.M.	870	0.83	27.5	32.2	19.2	0.7	691.8	79.6
6	Orally	1200	0.91	41.1	18.6	13.1	8.6	977.4	81.4
9	Orally	870	0.99	40.5	21.5	21.5	17.12	880.1	100.5
11	I.M.	500	1.04	49.5	16.8	9.3	7.6	415.9	83.2
12	I.M.	750	1.15	30.7	21.7	8.9	9.7	532.3	71.0
1	Orally	1168	1.2	26.9	11.0	4.8	3.7	542.0	46.4
20	I.M.	1000	1.77	19.48	10.06	7.95	6.35	438.4	43.8

¹ Intramuscularly.

that Najjar's ('41) suggestion of a relatively small test dose had merit and we have therefore adhered to his practice of giving 0.016 mg. per kilogram of body weight. It seemed to us that intravenous administration, which has been used by some investigators, is a decidedly unphysiological method and, therefore, we have for the most part used the intramuscular route.³ It will be noted that for the most part the same conclusion as to the riboflavin status of the individual was reached from the saturation test (assuming that figures below 35% excretion represent a deficiency) as from the analysis of a fasting sample. Of thirteen subjects with a fasting level less than 0.3 μ g. per milliliter, only three excreted more than 35% of the administered riboflavin; while in the group of twenty whose initial excretion was above 0.3 μ g. per milliliter, only three showed less than 35% excretion.

The coefficient of correlation between the fasting excretion expressed in micrograms per milliliter and the per cent of the test dose recovered within 4 hours is 0.61. This indicates a good degree of probability that the second figure depends on the first and that, in most instances, a correct conclusion will be drawn from an examination of only a fasting morning specimen. This saves considerable time as compared with the more laborious and time-consuming saturation test.

Examination of figure 1 will show that on the basis discussed above, a deficiency of riboflavin is a rather common finding and occurs to a greater or lesser degree in all the groups studied. Of the group of medical students, 25% were deficient as compared with 30% of the group of white draftees of approximately the same age but of differing economic status. Also, it is interesting that of a corresponding group of colored draftees, 50% showed a deficiency. A considerably higher percentage of the hospital patients had low values but that might have been anticipated because they were a highly selected group. Of the persons selected at random in the rural area about 65% showed a deficiency indicating, we believe, that a low intake of riboflavin is widespread throughout the general population in this area. We are well aware that single determinations of riboflavin excretion in the urine, such as we have carried out on many of our subjects, represent only isolated instances of a continually changing pattern. Subsequent analyses might have resulted in a different impression. However, when the figures are obtained from a rather large group of individuals, we feel that the finding of considerable deficiency is valid for the group

³ We are indebted to the Winthrop Chemical Company, Inc., Department of Medical Research, for the riboflavin (Flavaxin Soluble, Niphanoid) used in these experiments.

as a whole even though it may be only a temporary condition for the individual. A resume of our data from January 19th to May 25th by weeks indicates that the incidence of deficiency is evidently lessening somewhat. This probably reflects the greater availability of fresh foods but does indicate that, as yet at least, the rationing program has not noticeably decreased the amount of riboflavin available.

SUMMARY

1. The riboflavin content of 658 urine specimens has been determined.
2. Evidence is presented that the riboflavin per milliliter of urine is a more constant value than others which have been used in expressing riboflavin excretion.
3. Saturation tests seldom give more information than can be obtained by analysis of a fasting morning specimen.
4. There is a widespread incidence of riboflavin deficiency in this area.

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UNIDENTIFIED FACTOR(S) IN YEAST AND LIVER ESSENTIAL TO CURE OF ACHROMOTRICHIA IN DOGS ON SYNTHETIC DIETS

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TWO FIGURES

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This experiment with dogs was undertaken with two objectives in view. Frost, Moore, and Dann ('41) previously reported that factor(s) in liver besides pantothenic acid are essential to the cure and prevention of graying in rats. Dann, Moore and Frost ('42) reported later that p-aminobenzoic acid, biotin, and/or inositol were ineffective against graying in rats. The authors were particularly interested then in establishing whether graying could be produced in dogs receiving all of the available synthetic B-complex factors, including adequate pantothenic acid, and if so, whether the graying could be cured by liver and yeast. A further objective was to administer all of the B-complex vitamins except choline by injection to determine whether this form of administration would provoke a different response than oral administration.

Extensive studies by Schaeffer, McKibbin, and Elvehjem ('42a, '42b), and by McKibbin, Schaeffer, Elvehjem, and Hart ('42) indicated that classical synthetic diets based on casein and sucrose and supplemented with thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, and choline, will support good blood regeneration in phlebotomized adult dogs, but produce only erratic and suboptimal growth in puppies. More recently Lambooy and Nasset ('43) reported the further inadequacy of p-aminobenzoic acid and inositol to supplement the vitamins used by the Wisconsin workers in pup nutrition. The latter workers reported complete failure of their pups at 100 to 150 days unless yeast or liver concentrate were given. The following experiments are considered in light of the above reports.

EXPERIMENTAL

Six black puppies and one black spotted 2-month-old mongrel puppy were placed on one of the two diets shown in table 1. The pups, comprising six females and one male, were all in excellent condition and free from worms when the experiment was begun. Since there was one set of three littermates and another of two, it was possible to set up the experiment to study to advantage the effect of injection versus oral vitamin supplementation (table 2).

TABLE 1

Composition of diets and vitamin supplements.

	DIET I B-COMPLEX INJECTED	DIET II COMPLETE ORAL
Casein (S.M.A. vitamin-free)	19%	19%
Sucrose	73%	73%
Corn oil	3%	3%
Haliver oil	0.5%	0.5%
Salts U.S.P. I	4%	4%
CuSO ₄ · 5H ₂ O	0.004%	0.004%
ZnSO ₄ · 7H ₂ O	0.004%	0.004%
Choline chloride	0.67%	0.67%
l-cystine	0.1%
p-aminobenzoic acid	0.04%
Ascorbic acid	0.01%
i-inositol	0.009%
Thiamine chloride hydrochloride	0.1 mg./kg./day	0.00053%
Riboflavin	0.1 mg./kg./day	0.00053%
Nicotinamide	2.0 mg./kg./day	0.0106%
Pyridoxine hydrochloride	0.06 mg./kg./day	0.00032%
Ca-d (+)-pantothenate	0.2 mg./kg./day	0.00106%

TABLE 2

DOG ¹	ONSET OF GRAYING MONTH ON DIET	DEGREE OF GRAYING ²	GRAYING CURED BY ADDITION OF	BLOOD VALUES					
				Top levels before liver or yeast therapy			Top levels after liver or yeast therapy		
				H B.	R.B.C.	W.B.C.	H.B.	R.B.C.	W.B.C.
1 ♀	2	+++	3% Liver extract ³	14.5	4.9	10,000	17.7	8.5	10,500
2 ♀	2	+++	15% Brewer's yeast	13.8	5.4	10,300	14.6	6.5	14,650
3 ♀	11	+	Gray at death	15.1	5.5	10,000	(8.6) ⁴	(3.75)	(6900)
4 ♀	8	+	Gray at death	15.7	5.9	12,050	(9.7)	(5.1)	(7900)
5 ♂	6	+++	5% Liver extract ³	15.5	5.6	12,200	13.9	5.8	13,100
6 ♀	9	++	10% Brewer's yeast	13	4.2	6,850	16.8	6.8	14,350
7 ♀	5	+	Liver paste (1:20) 12 gm. per day	15	5.7	6,500	17.6	7.2	12,700

¹ Dogs 1 and 2 and dogs 3, 4, and 5 were littermates.

Dogs 1, 3, 5, 6, and 7 received synthetic B-complex vitamins by injection.

Dogs 2 and 4 received all supplements orally.

² +++ = gray over most of body, ++ = about half gray, + = graying slight but distinct.

³ Liver extract — fraction insoluble in 70% alcohol.

⁴ Values in parentheses are blood levels about 10 days prior to death.

Two pups were fed diet II (table 1), thus receiving all nourishment by mouth. The level of calcium pantothenate chosen both for injection and as per cent of the diet allows a daily intake of about twice the requirement figure stated by Schaeffer, McKibbin, and Elvehjem ('42a) for this compound in growing pups. The level of other vitamins, including choline, was also in accord with levels used by the Wisconsin workers. The levels of cystine, p-aminobenzoic acid, ascorbic acid, and inositol in diet II were arbitrary since they are not known to be needed by the dog.

The remaining five dogs were fed diet I (table 1) and the synthetic B-complex vitamins were administered in the following manner.

A sterile, isotonic solution made to contain (per milliliter of solution) 1 mg. thiamine HCl, 1 mg. riboflavin, 35 mg. nicotinamide, 0.6 mg. pyridoxine HCl, and 2 mg. calcium pantothenate, was injected subcutaneously three times weekly in amounts calculated to supply the per kilogram body weight allowance shown in diet I (table 1). The injection solution contained 0.5% boric acid as a preservative and was sterilized by filtration. The solution was filled into 10 ml. standard rubber-stoppered vials and stored in the cold to minimize destruction of thiamine and pantothenate. Tri-weekly injection of this solution was carried out easily and with no apparent ill effect to dogs for as long as 18 months.

Although the dogs on diet I, unlike those on diet II, did not get p-aminobenzoic acid, inositol, cystine, or ascorbic acid routinely, trials of p-aminobenzoic acid and inositol were made on the injected dogs, as will be detailed later. The presence of cystine and ascorbic acid in diet II did not appear to have nutritional significance for this experiment.

Since graying appeared first in the growing dogs at the nape of the neck and base of the tail, one of these areas was shaved prior to addition of supplement to the diet. With this device, the effect of the supplement on hair growth and pigmentation was apparent sooner than it otherwise would have been. Active supplements promoted both increased hair growth and repigmentation which were readily apparent in the shaved area in 4-6 weeks. When no supplement or an inactive supplement was given, hair growth was very slow and the new hair was predominantly gray. Photographs were made to supplement these observations. The photograph in figure 1 illustrates the marked depigmentation of new hair in a shaved area on a young dog receiving diet I.

Blood studies were made by standard methods on 5 ml. samples drawn from the radial vein into a 10 ml. syringe wetted with oxalate solution.



Fig. 1 Dog 2, showing typical graying on basal diets with marked graying of new hair in the shaved area at the nape of the neck.

RESULTS

Growth of dogs 1-6 is shown in figure 2. The growth record of dog 7 was uneventful and is not shown. The littermate female pairs, dogs 1 and 2, and dogs 3 and 4, grew at equal rate as seen in the figure. Although all the dogs grew well as to stature, they did not have the sleek, plump appearance of really well-nourished dogs. Supplements of liver extracts or yeast caused improvement in the general appearance of the treated dogs, but this was not accompanied by a weight increase in every case.

Littermate dogs, 3, 4, and 5, grew rapidly to maturity (fig. 2), but declined gradually after reaching the peak of the rapid growth period. Both the activity and appetite of these dogs appeared to lessen over a period of months. When severe anorexia set in, dog 5 was placed on liver extract therapy after which activity and appetite increased. Dogs 3 and 4 continued to lose weight to the point where they refused food entirely. They were sacrificed after about 2 weeks abstinence from food and following the onset of a bronchial infection which appeared in both dogs at about the same time.

Dog 6 (injected) alone showed a "saw-tooth" weight curve during the rapid growing period. This dog appeared to show growth recoveries alternately on a total injection of 250 μ g. of biotin in the form of a concentrate¹ from liver residue and a total injection of 60 mg. of p-amino-benzoic acid (fig. 2). These responses cannot be considered significant, however, since they occurred in only one of seven dogs. Furthermore,

¹ The concentrate, which contained 0.13% biotin on a dry basis, did not contain significant amounts of other known B-complex factors.

dogs which received p-aminobenzoic acid throughout showed no untoward differences from dogs which received none.

The time when graying was first seen in the different dogs ranged from 2 to 11 months. The degree of graying also varied considerably as shown in table 2. The time of appearance and the degree of graying were fairly constant among littermates and about equal for both basal diets.

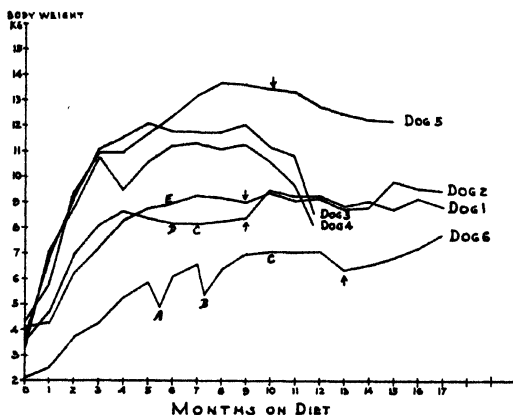


Fig. 2 Growth of dogs 1-6. Dogs 1, 3, 5, and 6 received diet I. Dogs 2 and 4 received diet II. At the points indicated by letters, the following injections were made. A 25 μ g. biotin (biotin concentrate) for 10 consecutive days; B, 20 mg. of p-aminobenzoic acid for 3 consecutive days; C, 0.5 mg. p-aminobenzoic acid plus 3 mg. inositol daily for 2 months; D, 0.5 mg. p-aminobenzoic acid daily for 1 month; E, 300 μ g. weekly of biotin for 3 months. At the points indicated by arrows, supplements of liver extract or yeast were added to the basal diets of dogs 1, 2, 5, and 6, as shown in table 2. Dogs 3, 4, and 5, were sacrificed at the points indicating the ends of their growth curves.

Dogs 1 and 6 received p-aminobenzoic acid (0.5 mg. per kilogram body weight) and inositol (3 mg. per kilogram body weight) daily by injection for 8 weeks during which no effect on hair growth or pigmentation were seen. Dog 2 received by injection 300 μ g. of biotin per week in concentrate form for 12 weeks, also without apparent effect.

All dogs except 3 and 4 were finally fed either brewer's yeast or liver extracts as shown in table 2. Appetite response and weight gain were better with 10% than with 15% brewer's yeast. Liver extract paste (1:20)² fed at 12 gm. per day (dog 7) gave a marked weight response and a clearcut cure of achromotrichia. The response to 5% alcohol insoluble liver fraction² was more rapid than with 3% of the same

² Liver extract paste (1:20) represents the fraction of a hot water extract of ground fresh liver which is soluble in 70% ethyl alcohol. The alcohol insoluble fraction is that fraction of the same hot water extract which is insoluble in 70% alcohol.

fraction. All dogs showed a clearcut demarcation of new black hair in the shaved area within 6 weeks after therapy was begun, and the dull, coarse texture of the entire fur coat gave way gradually to one of good texture and lustre. The displacement of gray hair from all parts of the body required 8-12 months therapy.

Blood studies were made at 2-week intervals during the latter part of the experiment. The maximum hemoglobin, erythrocyte and leucocyte levels attained before and following liver or yeast therapy are shown in table 2.

Blood value determinations indicated a low-grade anemia and leucopenia in some of the dogs before yeast or liver therapy with a response to high average values following 4-6 weeks of therapy. Dogs 3 and 4 showed a rather severe anemia shortly before death, but this may have been aggravated both by infection and inanition. A considerable increase in venous pressure with greater ease of blood withdrawal was noted consistently following yeast or liver therapy. Injection of p-aminobenzoic acid, inositol, or biotin concentrate had no measured effect on the blood picture.

The general improvement in blood picture on liver or yeast therapy ran parallel to an improvement in appetite, weight gain, increased rate of hair growth and repigmentation. A striking outward manifestation of nutritive betterment was also seen in improved muscle tone and spirit of the dogs soon after liver or yeast therapy was begun.

None of the female dogs came into heat while on the basal diets. Dog 6, which came into heat 3 months after addition of 10% yeast to diet I, was not bred successfully.

DISCUSSION

The above investigation substantiates the previous conclusion from this laboratory that yet unknown factor(s) in yeast and liver play an essential part in pigmentation of hair. Schaeffer et al. ('42b) and Lambooy and Nasset ('43) likewise reported achromotrichia in some of their dogs receiving adequate pantothenate. In work with chicks McGinnis, Norris, and Heuser ('42) reported evidence that a factor other than pantothenate supplied by yeast is essential to normal feather pigmentation. Similar observations have been made in this laboratory.

Biotin and p-aminobenzoic acid have failed consistently to demonstrate chromatrichial activity in many trials in rats, dogs, and chicks in this laboratory. Emerson and Keresztesy ('42), and Emerson ('41) have reported the inefficacy of these compounds in rats. Martin ('42) reported that a "folic acid" concentrate prepared from yeast contains

a chromotrichia factor for sulfaguanidine poisoned rats. It should be noted in this regard that Briggs, Luckey, Elvehjem, and Hart ('43) have subdivided the "folic acid" concentrate described originally by Hutchings, Bohonos, and Peterson ('41) into three separate factors for the chick, any or all of which might conceivably be achromotrichia factors for various species.

There is much accumulated evidence in the literature (Langston et al., '38; Wilson et al., '42; O'Dell and Hogan, '43; and Waisman and Elvehjem, '43) to show that important hematopoietic factor(s) for the monkey and chick are yet to be identified in yeast and liver. This investigation indicates that such factors may also be important in the nutrition of the dog.

The level of choline used in this study provides a somewhat higher intake than that provided by Schaeffer, Elvehjem and Hart ('42b), i.e. 50 mg. per kilogram per day. The low level of choline used by Lambooy and Nasset ('43), i.e. 0.2. mg. per kilogram per day, leaves a question as to whether this might not be the cause of failure in their dogs. As pointed out by the latter workers, however, the high level of 35% casein used by them might be expected to modify the choline requirement. Both of the above groups have pointed to possible impurity of unextracted sucrose and casein as limiting factors in obtaining severe deficiencies in growing dogs.

SUMMARY

Pups fed purified diets received synthetic B-complex supplements orally or by injection throughout the experiment. The response to both types of vitamin administration appeared to be equal.

The growing dogs receiving thiamine, riboflavin, nicotinamide, pantothenate, pyridoxine, and choline developed achromotrichia and decreased hair growth in 2 to 11 months on the diet. Two of the dogs finally failed after reaching maturity on the synthetic diet. Inositol, p-aminobenzoic acid, or biotin were ineffective in changing the course of the deficiency, whereas liver fractions or whole dried yeast brought about complete cures.

General improvement in body tone and in the blood picture ran parallel to the cure of achromotrichia in the dogs which received yeast or liver.

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RETENTION OF THE B-VITAMINS IN RARE AND WELL-DONE BEEF¹

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The vitamin content of meat usually has been reported on the raw basis but meat is seldom eaten raw. Heat losses during cooking may be expected since thiamine is heat labile and in the presence of moisture (Frost, '43) pantothenic acid is also. Thiamine, pantothenic acid, riboflavin, and nicotinic acid are water soluble and no doubt are lost to some extent in the drippings from roasts. Rare roast beef is cooked to a lower internal temperature than well-done beef and has lower weight losses in the oven. Since these factors may affect the retention of the B-vitamins, it seemed desirable to determine the losses of thiamine, riboflavin, nicotinic acid and pantothenic acid in rare and well-done beef, roasted by a method which is not only suitable for use in the home but could be controlled carefully enough for research purposes.

EXPERIMENTAL

The roasts in this study contained two ribs each. They were the 7-8th, 9-10th, and 11-12th.³ The roasts were cut from paired wholesale ribs from carcasses weighing approximately 400 to 500 pounds. The chine bone was not removed nor was the meat loosened from the spinal processes.

A method of roasting beef for home use has been recommended by The Committee on Preparation Factors, National Cooperative Meat Investigations ('42). It gives low weight losses in the oven as well as high palatability and appears to offer reasonably good conditions for

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² Cooperative project between the Division of Rural Home Research and the Nutrition Laboratory, Department of Animal Husbandry.

³ The ends of the ribs were removed beginning to cut at a point 1 inch below the ribeye at the twelfth rib and cutting parallel with the rib ends; then the individual roasts were made with the knife crowding the rear edge of the twelfth, the rear edge of the tenth, the rear edge of the eighth, and the rear edge of the sixth ribs.

retaining the B-vitamins. It was modified somewhat for use in this investigation. The roasts were removed from the refrigerator at a roast temperature of 7–10°C. and cooked on their sides on a rack in an uncovered pan without water, salt or flour and at a constant oven temperature of 150°C. until the internal temperature of the meat reached 80°C. for the well-done roasts and 60°C. for the rare roasts. The internal temperature of well-done roasts does not rise after removal from the oven but that of rare roasts does. In preliminary studies it was found impossible to predict the extent of this rise, so that a uniform maximum could be attained. Therefore, the internal temperature on removal from the oven was standardized instead. The

TABLE 1
Cooking data, moisture and ether extract.

U. S. CARCASS GRADE	NUMBER OF ROASTS		TIME IN OVEN	WEIGHT LOSSES IN OVEN		MOISTURE	ETHER EXTRACT
				Volatile ¹	Drippings ²		
			Hours	%	%	%	%
Choice							
Raw	6	Mean	52.9	31.2
		Range	49.9–56.0	28.0–34.5
Commercial							
Raw	18	Mean	62.9	16.7
		Range	56.8–68.7	8.5–24.8
Rare	9	Mean	1.9	9.0	2.2	57.8	18.0
		Range	1.6–2.5	7.4–10.3	0.8–3.7	53.6–63.7	9.9–24.3
Well-done	9	Mean	3.2	16.6	4.5	54.3	19.9
		Range	2.5–3.9	12.8–21.7	2.8–7.3	49.2–59.7	15.1–25.6

¹ Mainly water.

² Includes fat, moisture, and hardened crusts.

rise after removal varied from 2–6°C. After cooking, the roasts were weighed and weight losses calculated (table 1). The rare roasts were allowed to stand at room temperature for 30 minutes or until the internal temperature began to fall, whichever was the longer. The well-done roasts were allowed to stand until the internal temperature reached 70°C.

The drippings were transferred to a graduated cylinder, the fat floated off the top by the addition of hot water, and the volume adjusted to 100 ml. Vitamin analyses were made by the methods used for the meat samples.

Meat samples for vitamin determinations were obtained from right and left roasts cut alike and used as pairs, one raw and the other cooked.

After the meat had been separated from the bone, the fat and lean together were passed through a household meat grinder twice and after thorough mixing, a portion was passed through a tissue mincer (SeEVERS and SHIDEMAN, '41). The resulting homogenous mince was mixed thoroughly before the samples were weighed. Details of the extraction procedure were given by McLaren et al. ('44).

Analyses of left and right raw rib roasts were made from the same carcass to test the assumption that their vitamin contents would be similar enough so that they could be used as pairs. The results show that the assumption is correct within the limits of experimental error (table 3, U. S. Choice carcass). Nicotinic acid shows more variability than do the other vitamins.

Moisture and ether extract were determined on the same sample (table 1). Approximately 2 gm. of the minced sample were weighed by difference, placed in dry ether extracted filter papers, and then in dry moisture dishes. They were dried in a vacuum oven at 95°C. for 48 hours. The dry samples then were placed in a soxhlet extraction apparatus and extracted with ether for 16 hours. Several of the meat samples could be placed in the same extraction chamber.

Calculations of retention were made as follows: Dry fat-free weight per 100 gm. of raw and cooked meat was calculated and the vitamin content expressed on the dry fat-free basis. The percentage retention was then calculated from these values. The percentage recovered in the drippings was based on the total content in the roast before cooking. Since this roast was not sampled for analysis before cooking the content before cooking was calculated by proportion from the content after cooking and the percentage retention.

THIAMINE

Methods

Thiamine was determined by two methods, that of Hennessy ('42) and that of Harris and Wang ('41). The Hennessy method was modified as suggested by McIntire et al. ('42) and Hinman.⁴ Slight modifications were made in the Harris procedure. A photofluorometer was used and the fat and lean were ground together before sampling. After digestion the fat was removed with chloroform. The Harris method gave more consistent recoveries than did the Hennessy. Only the Harris values, therefore, are given in the tables.

⁴ Personal communication from Winifred F. Hinman of the University of Chicago.

Content

The thiamine content of the eighteen raw rib roasts from the Commercial carcasses averaged 1.3 $\mu\text{g./gm.}$ by the Harris method (table 3) and 0.8 $\mu\text{g./gm.}$ by the Hennessy method. The individual Harris values were from 0.1 to 0.9 $\mu\text{g./gm.}$ higher than the corresponding Hennessy values.

Similar thiamine values were found in the three raw roasts from the same carcass. Pyke ('40) found that thiamine was not evenly distributed between the muscles of the individual animal, ranging in swine from 85 I. U. per 100 gm. in the tongue to 300 in the psoas. Since it is known that the weight relationships of the individual muscles change with the cut of rib, it would seem that all of the muscles in these three beef cuts must have similar thiamine contents. This was confirmed by separate analyses of ribeye and other lean tissue from these rib cuts (table 2). The finding of no difference between the muscles in the rib of beef does not eliminate the possibility of difference between other muscles of the beef carcass.

TABLE 2

Thiamine content as related to muscle composition of raw rib roasts¹
(Micrograms per gram)

RIBS IN ROAST	ANIMAL A.		ANIMAL B.		ANIMAL C.		ANIMAL D.		ANIMAL E.	
	Ribeye	Other lean	Ribeye	Other lean	Ribeye	Other lean	Ribeye	Other lean	Ribeye	Other lean
11-12th	2.8	2.6	2.7	2.7	2.0	1.8	2.5	2.5	2.6	2.5
9-10th	2.6	2.5	2.7	2.6	1.9	1.7	2.5	2.4	2.6	2.5
7-8th	2.6	2.5	2.7	2.5	1.9	1.9	2.5	2.5	2.5	2.6

¹ These analyses were made on lean tissue from which the fat had been removed. The values should be higher than when the fat is included in the sample.

Animal variability is indicated by the wide range in thiamine content of the raw roasts from the Commercial carcasses (table 3). Statistical treatment showed that the difference between animals was highly significant. Fatness may not influence thiamine content of beef if fat and lean are not separated since the thiamine content of the raw roasts from the Choice carcass fell within the range of values for the roasts from the Commercial carcasses (two grades below Choice). Whether this is the result of an increase in the thiamine content of lean or whether the extra fat (table 1) contained an appreciable amount of thiamine was not determined.

In the literature, thiamine values of 1.7 and 2.8 $\mu\text{g./gm.}$ were reported by Waisman and Elvehjem ('41) for beef round; of 1.14 $\mu\text{g./gm.}$ for top round by Booher and Hartzler ('39); and of 0.63 $\mu\text{g./gm.}$ for beef

round by Cheldelin and Williams ('42). The range of 0.8 to 1.7 $\mu\text{g./gm.}$ found in beef ribs in the present study is intermediate between these values for round. Since beef round contains such a large number of muscles, muscle variation cannot be excluded, but it is suggested that animal variation may account for some of the differences in thiamine content of beef reported in the literature.

Cooked roasts of beef in the present study showed the following thiamine content in micrograms per gram: by the Harris method, rare 1.2 (range 1.1–1.5), well-done 1.1 (range 0.8–1.4); by the Hennessy method, rare 0.9 (range 0.7–1.1), well-done 0.7 (range 0.5–0.9).

Retention

Retention of thiamine averaged 75% in rare roasts and 69% in well-done roasts (table 4). Retentions for the three roasts within a carcass were quite similar. The difference in retention between rare and well-done roasts was small but statistically was highly significant. Differences between rare and well-done roasts were observed in maximum internal temperature, time in oven, and weight losses in the oven (table 1), all of which may have influenced thiamine retention.

Waisman and Elvehjem ('41) reported 44 and 45% retention in two samples of roasted beef round but gave no details of their method of roasting. Since retention in their samples was considerably below those obtained in the present study, it may be assumed that their cooking conditions were less favorable for thiamine retention. Later, the Wisconsin workers (McIntire et al., '42) considered that in their previous work on retention the methods of cooking and preparation of the samples had not been properly standardized. Using a method of cooking similar to the one used in the present study, they obtained in roast pork an average retention of 70% of the thiamine. This agrees well with the average retention of 69% found here for well-done beef.

Retention in the drippings in the present study was only a small percentage of the total, but the drippings were so dark in color that the tests were not entirely satisfactory. Even adsorption failed to take out all of the color. The large amount of evaporation which occurred is indicated by the volatile losses (table 1). The roasting pan contained no added water or fat and the amount of each in the drippings was so small that tiny pointed crusts were formed where the meat juices dripped into the pan. By the end of the cooking period they were dry and hard and remained insoluble even when papain was used for digestion. Under such conditions little thiamine retention could be expected. The Wisconsin workers (McIntire et al., '42) also reported small recoveries from the drippings of pork roasts.

RIBOFLAVIN

Methods

Riboflavin was determined on papain-takadiastase extracts from which the fat had been removed with chloroform, and the proteins by precipitation at pH 6.8-7.0. Two methods of determining the vitamin were used, a simplified fluorometric method (McLaren, et al., '44), and the microbiological procedure of Snell and Strong ('39) with modifications in the preparation of the sample to eliminate the effect of foreign growth stimulants.

TABLE 3
Vitamin content of the raw rib roast of each pair.

ROAST NUMBER	RIBS IN ROAST	MICROGRAMS PER GRAM MOIST BASIS				
		Thiamine (Harris)	Riboflavin		Nicotinic acid	Pantothenic acid
			Fluoro- metric	Micro- biological		
U. S. Choice Carcass						
1059 L	11-12th	1.3	1.1	1.2	33	3.1
1059 R	11-12th	1.3	1.1	1.2	32	3.0
1060 L	9-10th	1.4	1.1	1.3	29	3.2
1060 R	9-10th	1.3	1.0	1.2	32	3.1
1061 L	7-8th	1.3	1.1	1.2	28	3.1
1061 R	7-8th	1.2	1.1	1.3	34	3.1
	Mean	1.3	1.1	1.2	31	3.1
	Range	1.2-1.4	1.0-1.1	1.2-1.3	28-34	3.0-3.2
U. S. Commercial Carcasses						
1041 R	11-12th	1.4	1.9	1.9	39	5.4
1042 R	9-10th	1.4	1.7	1.6	44	5.0
1043 R	7-8th	1.4	2.0	1.8	41	5.7
1047 L	11-12th	1.4	1.8	1.9	45	5.6
1048 L	9-10th	1.4	2.0	1.9	44	5.5
1049 L	7-8th	1.2	2.2	2.0	50	5.6
1053 L	11-12th	1.0	1.0	1.2	42	4.4
1054 L	9-10th	1.1	1.2	1.3	47	5.0
1055 L	7-8th	0.8	1.3	1.6	47	5.3
1044 L	11-12th	1.3	1.4	1.4	59	3.8
1045 L	9-10th	1.3	1.5	1.6	51	5.1
1046 L	7-8th	1.4	1.5	1.6	52	4.6
1050 R	11-12th	1.7	1.4	1.5	59	4.2
1051 R	9-10th	1.7	1.3	1.5	60	4.3
1052 R	7-8th	1.7	1.3	1.5	55	5.3
1056 L	11-12th	1.3	1.1	1.5	58	4.7
1057 L	9-10th	1.3	1.1	1.5	50	5.0
1058 L	7-8th	1.3	1.3	1.4	47	4.8
	Mean	1.3	1.5	1.6	49	4.9
	Range	0.8-1.7	1.0-2.2	1.2-2.0	39-60	3.8-5.7

TABLE 4
Retention

RARE				WELL-DONE			
Dry fat-free basis		Retained	Recovered	Dry fat-free basis		Retained	Recovered
Raw	Cooked	in meat ¹	in drippings ¹	Raw	Cooked	in meat ¹	in drippings ¹
μg./gm.	μg./gm.	%	%	μg./gm.	μg./gm.	%	%
Thiamine ²							
5.99	4.77	80	3	6.79	4.76	70	..
6.26	5.00	80	2	7.04	4.62	66	..
7.00	5.51	79	2	7.07	5.07	72	..
6.96	5.22	75	1	6.54	4.72	72	2
7.19	5.31	74	3	6.62	4.66	70	1
7.28	5.19	71	1	6.30	4.28	68	1
6.44	4.74	74	2	5.65	3.81	67	2
7.00	5.03	72	2	5.71	3.54	62	1
7.13	5.23	73	2	4.13	2.96	72	2
	Mean	75	2		Mean	69	
	Range	71-80	1-3		Range	62-72	
Riboflavin (fluorometric)							
7.08	5.89	83	..	9.99	7.54	75	..
7.89	6.11	77	..	9.66	7.24	75	..
8.14	6.51	80	..	10.71	7.86	73	..
6.59	4.93	75	..	10.10	7.53	75	..
6.88	5.52	80	..	11.27	8.28	73	..
6.57	5.38	82	..	12.96	11.28	87	..
6.24	5.61	90	..	6.57	5.44	83	..
7.51	5.85	78	..	7.19	5.65	79	..
7.34	7.33	98	..	7.65	5.87	77	..
	Mean	83			Mean	77	
	Range	75-98			Range	73-87	
Nicotinic acid							
269.6	222.4	83	5	190.1	139.3	73	..
244.3	202.0	83	4	221.3	148.6	67	..
267.3	199.3	75	5	204.5	137.9	67	..
246.9	173.7	70	3	215.0	185.2	86	15
260.7	171.8	66	6	214.1	189.7	88	7
244.3	192.5	79	4	261.5	168.8	65	7
293.8	209.2	71	3	229.1	208.0	91	9
265.1	187.4	71	5	249.7	219.1	88	7
252.2	191.1	76	5	246.3	212.6	86	8
	Mean	75	4		Mean	79	
	Range	66-83	3-6		Range	65-91	
Pantothenic acid							
17.5	16.3	93	4	26.3	21.0	80	..
24.3	21.9	90	2	25.0	19.3	78	..
23.7	22.4	95	3	28.5	20.3	71	..
17.7	15.7	89	2	27.0	18.5	69	3
18.4	17.1	93	3	27.0	18.6	69	4
23.2	20.8	90	2	29.3	20.5	70	2
23.4	20.0	85	1	23.7	20.3	86	3
26.3	23.4	89	1	26.5	19.9	75	1
24.7	23.5	95	1	27.9	20.6	74	1
	Mean	91	2		Mean	75	2
	Range	85-95	1-4		Range	69-86	1-4

¹ Percentage of total in roast before cooking.

² Percentage thiamine retained in roast beef by the Hennessy method of analysis averaged for rare, 96% (range 73-128%) and for well-done, 61% (range 50-74%).

Content

The riboflavin content of the eighteen raw rib roasts from the Commercial carcasses averaged 1.5 and 1.6 $\mu\text{g./gm.}$ by the fluorometric and microbiological methods respectively (table 3).

Similar riboflavin values were obtained in the three raw roasts from the same carcass. Peterson, Brady, and Shaw ('43) have shown that the riboflavin content of pork varies with the muscle tested, ranging in the same pigs from 1.3 $\mu\text{g./gm.}$ in longissimus dorsi to 3.0 $\mu\text{g./gm.}$ in triceps brachii. The data in the present study indicate that all of the muscles in the rib section of beef probably have similar riboflavin values since in spite of the change in the proportions of the muscles present in the different rib cuts similar riboflavin values were obtained for them. This does not eliminate the possibility that other muscles of beef may show considerable variation.

Animal variability is indicated by the range of riboflavin values for the raw roasts from the Commercial carcasses (table 3). Statistical analyses showed the difference between animals to be highly significant. This was true for data from both methods of determining riboflavin. The riboflavin values of the raw roasts from the Choice carcass fell within the range of those from the Commercial carcasses, suggesting that fatness of the animal may have little influence on riboflavin content of beef if the fat and lean are not separated. It is not known whether this resulted from an increase in the riboflavin content of lean or whether the extra fat contained an appreciable amount of riboflavin, as was found in pork fat by Peterson et al. ('43).

Munsell ('41) reported riboflavin values for beef of 1.35 and 1.45 $\mu\text{g./gm.}$ in eye muscle of rib. The average of 1.5 $\mu\text{g./gm.}$ for the eighteen rib roasts from the Commercial carcasses seems to agree with Munsell's values but our values were obtained on the mixture of fat and lean and hers on the ribeye only. Waisman and Elvehjem ('41) found 1.9, 2.4, and 3.5 $\mu\text{g./gm.}$ in beef round; Cheldelin and Williams ('42) 2.2 $\mu\text{g./gm.}$ in beef round; and Munsell ('41) 2.14 and 2.22 $\mu\text{g./gm.}$ in top round. Many of these values for round are similar to the higher ones for beef ribs in the present study.

Cooked rib roasts of beef in the present study showed the following riboflavin content in micrograms per gram: by the fluorometric method, rare 1.2 (range 1.1–1.4), well-done 1.7 (range 1.2–2.5); by the microbiological method, rare 1.4 (range 1.3–1.6), well-done 2.0 (range 1.4–2.9).

Retention

Retention of riboflavin averaged 83% in rare and 77% in well-done roasts by the fluorometric method. The difference in retention between rare and well-done was small and not significant by either method. Since retentions by the two methods of analysis did not differ significantly, only the fluorometric values are given (table 4). The Wisconsin workers (McIntire et al., '42) reported retention of 85% in roast pork which agrees reasonably well with the values for roast beef obtained in the present study.

In the drippings of the present study the dark color prevented satisfactory fluorometric readings. Recovery by the microbiological method (approximately 5%) did not account for all of the riboflavin lost from the roast during cooking. The hardened crusts which remained insoluble may have contained appreciable quantities which could not be measured. The Wisconsin workers (McIntire et al., '42) recovered 5-10% in the drippings of their pork roasts which probably accounted for most of the remainder of the riboflavin in their raw meat.

NICOTINIC ACID

Method

Nicotinic acid was determined on papain-takadiastase extracts from which the fat had been removed with chloroform and the proteins by precipitation at pH 6.8-7.0. The microbiological method of Snell and Wright ('41) was used.

Content

The nicotinic acid content of the eighteen raw roasts from the Commercial carcasses averaged 49 $\mu\text{g./gm.}$ (table 3). Considerable variation was observed between roasts within a carcass but since it was not statistically significant it indicates no difference in the nicotinic acid content with variation in muscle composition of the roasts. Animal variation in nicotinic acid content was shown to be highly significant by statistical treatment. That fatness may influence the nicotinic acid values is shown by the lower values obtained in the Choice carcass.

Dann and Handler ('42) reported nicotinic acid values of beef ribs to be 66, 73, and 82 $\mu\text{g./gm.}$ by their chemical method. These values are somewhat higher than the ones obtained in the present study. However, they gave no data to indicate the fatness of the meat used. They reported a range of 31 to 82 $\mu\text{g./gm.}$ for a large number of beef muscles.

The values 39 to 60 $\mu\text{g./gm.}$ obtained in the present study fall within this wider range. They observed considerable variation between samples and attributed it to variation among individuals of the species, but suggested that some of it may have been due to the effects of ageing, over which they had no control. In the present study ageing could not be standardized but roasts 1041, 1042, and 1043 were stored the longest.

Cooked rib roasts of beef in the present study gave nicotinic acid values in micrograms per gram of 47 (range 41–53) for rare and of 46 (range 35–57) for well-done.

Retention

Retention of nicotinic acid in rare roasts averaged 75%, and in well-done roasts 79% (table 4). The difference in retention between rare and well-done roasts was small and not significant. Recovery in the drippings was too low to account for all of the nicotinic acid lost from the meat. Perhaps some of the nicotinic acid in the hardened drippings was not recovered.

The Wisconsin workers (McIntire et al., '42) obtained an average nicotinic acid retention of 85% in roast pork which agrees reasonably well with 79% in roast beef in the present study. The Wisconsin workers recovered 5–15% in the drippings which accounted for most of the remainder of the nicotinic acid in their raw meat. However, their pork drippings contained a large amount of fat and the drippings were not dry or hard. Dann and Handler ('42) reported losses during cooking of some meats but did not include roasts of beef. None of their methods of cooking was described. Perhaps loss of juices from the meats during cooking may have been sufficient to account for most of what they called surprisingly large and inexplicable losses.

PANTOTHENIC ACID

Method

Pantothenic acid was determined on papain-takadiastase extracts from which the fat had been removed with chloroform and the proteins by precipitation at pH 6.8–7.0. The microbiological procedure of Strong et al. ('41) was used with the foregoing modifications in the preparation of the sample to eliminate the effect of foreign growth stimulants.

Content

The pantothenic acid content of the eighteen raw rib roasts from the Commercial carcasses averaged 4.9 $\mu\text{g./gm.}$ (table 3). There was

considerable variation between roasts in the same carcass but it was not significant. This indicates that there was little change in pantothenic acid content with variation of the individual muscles in the rib section of beef. Animal variability, however, was statistically significant. Fat may be one cause of animal variability since the values for the Choice carcass were below those for the Commercial carcasses.

Waisman and Elvehjem ('41) reported pantothenic acid values of 2.1 to 10.0 $\mu\text{g./gm.}$ and Cheldelin and Williams ('42) reported 4.7 to 5.1 $\mu\text{g./gm.}$ These values are for beef round but compare reasonably well with 3.8 to 5.7 $\mu\text{g./gm.}$ for the beef ribs of this study.

Cooked rib roasts of beef in the present study showed pantothenic acid values in micrograms per gram of 4.8 (range 3.9–5.5) for rare and of 5.1 (range 4.6–5.6) for well-done.

Retention

Retention of pantothenic acid averaged 91% in rare roasts and 75% in well-done roasts (table 4). The difference between rare and well-done roasts was highly significant by statistical analysis. Recovery in the drippings was less than 5%. In well-done beef, it is too low to account for the remainder of the pantothenic acid.

Frost ('43) reported that while no destruction of moisture-free pantothenate occurred on heating at 120°C. for 40 hours, the presence of traces of moisture permitted slight hydrolysis at 60°C. In the presence of moisture, he found the rate of destruction to be a function of temperature. In view of Frost's findings, the internal temperature of the rare roasts may cause some destruction but the higher internal temperature of the well-done roasts should increase the destruction, and little pantothenic acid should be left in the drippings. The results of the present study, therefore, are in agreement with Frost's observations.

CONTRIBUTION OF BEEF ROASTS TO DAILY VITAMIN ALLOWANCE

The recommended daily allowances for specific nutrients set up by the Committee on Food and Nutrition of the National Research Council ('43) for a moderately active woman or a sedentary man were used as the basis for calculating the percentage supplied by one serving of roast beef. The size of the serving was the customary 4 ounces on the raw basis. When roasted by the method used here, one serving would supply approximately 7% of the thiamine, 6% of the riboflavin, and 37% of the nicotinic acid. Allowances for pantothenic acid have not been recommended.

SUMMARY

Right and left 2-rib roasts of beef were cut alike and used as pairs, one being analyzed raw and the other after cooking by a standardized method. The entire meat in each roast was ground and samples used for the determination of thiamine, riboflavin, nicotinic acid, and pantothenic acid.

The average vitamin content of the eighteen raw roasts from the Commercial carcasses was in $\mu\text{g./gm.}$: thiamine 1.3; riboflavin 1.5 and 1.6 by the fluorometric and microbiological methods, respectively; nicotinic acid 49; and pantothenic acid 4.9. Differences in vitamin content between the raw rib roasts within a carcass were not significant for any of the four vitamins but differences between animals were highly significant for thiamine, riboflavin, and nicotinic acid and significant for pantothenic acid.

Thiamine and riboflavin values for the Choice carcass were within the range of those from the Commercial carcasses (two grades below Choice) but the Choice carcass was lower than the Commercial carcasses in nicotinic acid and pantothenic acid.

Retentions in rib roasts of beef, rare and well done respectively, were: thiamine, 75 and 69%; riboflavin, 83 and 77%; nicotinic acid, 75 and 79%; pantothenic acid 91 and 75%. Retentions of thiamine and pantothenic acid were significantly lower in the well-done than in the rare roast but with riboflavin and nicotinic acid the differences between rare and well-done roasts were not significant.

One serving of rib roast of beef was calculated to furnish approximately 7% of the thiamine, 6% of the riboflavin and 37% of the nicotinic acid recommended for a moderately active woman for 1 day.

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COMPARISON OF RESPONSE OF TURKEY POULTS AND OF CHICKS TO DIFFERENT FORMS OF VITAMIN D¹

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The vitamin D requirement of turkey poults has been reported as 60 to 70 International Units per 100 gm. of feed by Baird and Greene ('35), 200 A.O.A.C. Units per 100 gm. by Jukes and Sanford ('39) and 80 A.O.A.C. Units per 100 gm. by Hammond ('41) and by Carver and Rhian ('42). Baird and Greene ('35) used a fortified cod liver oil in determining the requirement not only of poults, but also of chicks and young pheasants. Their results with chicks would indicate the potency of this oil in A.O.A.C. Units to be the same as its potency in International Units. Jukes and Sanford ('39) used U.S.P. Reference Cod Liver Oil and also a fish oil blend, and reported that a given number of A.O.A.C. Units of the latter was 1.4 times as effective for poults as the same number of A.O.A.C. Units of the former. This would indicate a difference in the ability of chicks and poults to use different forms of vitamin D similar to the difference long known to exist between chicks and rats. Such a difference would of course help to explain differences in requirements determined in different laboratories.

Hammond ('41) found no difference between U.S.P. Reference Cod Liver Oil and a fortified cod liver oil. Carver and Rhian ('42) used a fortified cod liver oil in confirming the requirement reported by Hammond ('41). In both these investigations Activated Animal Provitamin D was also fed, but at levels which proved too high to permit accurate assessment of its potency. More recently Boucher ('44) reported that a given number of A.O.A.C. Units of vitamin D of irradiated 7-dehydrocholesterol or irradiated animal provitamin had much greater potency for turkey poults than did the same number of A.O.A.C. units of vitamin D of U.S.P. Reference Cod Liver Oil or a fortified sardine oil. The latter contained no vitamin D from any irradiation product and differed

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but slightly from Reference Cod Liver Oil with respect to the effectiveness for turkeys of a given number of A.O.A.C. Units. In the past the levels of vitamin D carriers used in turkey feeds have been universally based on the assumption that chickens and turkeys are alike in their ability to utilize different forms of vitamin D. Since irradiation products are being manufactured and used in poultry feeds in large quantities, the results reported by Boucher ('44) are of considerable practical importance, and an attempt to confirm them seemed to be warranted. It also seemed desirable to establish the potency of a commercial cod liver oil in terms of A.O.A.C. Units and test this material on poults in direct comparison with irradiation products whose efficacy for chicks had been established.

PROCEDURE

Some difficulty was encountered in obtaining poults for this work, and it was necessary to divide the investigation into two experiments. On June 23, 1943, 178 Broad Breasted Bronze poults were obtained from a commercial hatchery and divided into thirteen groups as indicated in table 1. Ten poults were started in group 1, eleven poults in groups 2 to 5, and fifteen or sixteen poults in each of the other groups.

TABLE 1
Response of turkey poults to different forms of vitamin D.
Experiment 1

GROUP	VITAMIN D SOURCE	LEVEL FED, A.O.A.C. UNITS PER 100 GM.	AVG. WT. OF POULTS IN GM. AT 4 WEEKS	EFFICIENCY TO 4 WEEKS, GM. GAIN PER GM. FEED	PER CENT OF BONE ASH AT 4 WEEKS	POTENCY OF VITAMIN D SOURCE, "POULT UNITS" PER GM. OIL
1	None	24.02 ¹	..
2	Reference cod liver oil, no. 2	80	289	0.448	32.65	115
3	Reference cod liver oil, no. 2	120	342	0.485	37.63	115
4	Reference cod liver oil, no. 2	160	391	0.528	39.21	115
5	Reference cod liver oil, no. 2	200	379	0.498	42.95	115
6	Cod liver oil, no. 8-117	72	278	0.442	32.53	200
7	Cod liver oil, no. 8-117	108	350	0.487	39.76	267
8	Cod liver oil, no. 8-117	144	356	0.484	40.00	204
9	Cod liver oil, no. 8-117	180	394	0.480	43.72	205
average						219
10	Irradiated 7-dehydrocholesterol	41	227	0.376	34.99	1060
11	Irradiated 7-dehydrocholesterol	61	338	0.465	38.30	954
12	Irradiated 7-dehydrocholesterol	82	351	0.487	41.27	880
13	Irradiated 7-dehydrocholesterol	102	362	0.421	43.91	828
average						931

¹ Bone ash at 3 weeks of age. Two surviving poults.

On June 30th, 140 poults hatched from eggs produced on the University poultry farm were divided into nine groups as indicated in table 2. Of this hatch, seventy poults were of the small White breed developed at the Beltsville Research Center and seventy were black poults, being a mixture of pure-bred Blacks, Black x White crossbreds, and White x Black crossbreds. Equal numbers of black and of white poults were placed in each group. Twelve poults were started in group 18 and sixteen poults in each of the other groups.

TABLE 2
Response of turkey poults to different forms of vitamin D.
Experiment 2

GROUP	VITAMIN D SOURCE	LEVEL FED. A.O.A.C UNITS PER 100 GM.	AVG. WT. OF POULTS IN GM. AT 4 WEEKS	EFFICIENCY TO 4 WEEKS. GM. GAIN PER GM. FED	PER CENT OF BONE ASH AT 4 WEEKS	POTENCY OF VITAMIN D SOURCE. "POULT UNITS" PER GM. OIL
14	Irradiated animal provitamin	48	313	0.428	36.90	760
15	Irradiated animal provitamin	72	345 ¹	43.57	1094
16	Irradiated animal provitamin	96	359 ¹	45.00	910
17	Irradiated animal provitamin	120	332	0.455	45.99	784
						average 887
18	None	..	173	0.341	30.35	
19	Reference cod liver oil, no. 2	80	327	0.441	37.28	115
20	Reference cod liver oil, no. 2	120	331	0.455	41.29	115
21	Reference cod liver oil, no. 2	160	347	0.478	43.68	115
22	Reference cod liver oil, no. 2	200	369	0.483	44.89	115

¹ Portion of feed lost.

The basal diet used in these experiments had the following composition: ground yellow corn 29, wheat flour middlings 10, wheat bran 10, pulverized heavy oats 10, commercial acid precipitated casein 6, soybean oil meal 12, dried skimmilk 8, dried brewers' yeast 6, dehydrated alfalfa leaf meal 5, calcium carbonate 1, defluorinated superphosphate 1.5, salt 0.5, Wesson oil 2. The basal mash was analysed for calcium and phosphorus using the methods recommended by the Association of Official Agricultural Chemists ('40) for plant materials. The mash was found to contain 1.06% calcium and 0.82% phosphorus. Since the carotene content of the alfalfa leaf meal was only 55 parts per million at time of assay, beta carotene in Wesson oil was added to this diet at the rate of approximately 800 µg. per pound. The Wesson oil so added and also the vitamin D oils added replaced equivalent quantities of the Wesson oil of the formula. The cod liver oil tested for its comparative efficacy on poults and chicks in this experiment was selected as repre-

sentative of that used as a vitamin D supplement in mixed poultry feeds. The levels of the cod liver oils were calculated to furnish, respectively, 80, 120, 160, and 200 A.O.A.C. Units of vitamin D per 100 gm. of feed, and the levels of the irradiation products were calculated to furnish, respectively, 40, 60, 80, and 100 A.O.A.C. Units per 100 gm. However, the diets were made up before all the chick assay results were available. On the basis of the chick assay results, which are summarized in table 3, the number of A.O.A.C. Units of vitamin D per 100 gm. of each of the experimental diets is shown in tables 1 and 2. Chick assay number 1 was made by an independent commercial laboratory; numbers 2 and 3 were made in the laboratories of E. I. Du Pont de Nemours and Co.

TABLE 3
Assays of vitamin D sources with chicks and poults.

	COD LIVER OIL, NO. 8-117	IRRADIATED 7-DEHYDROCHOLESTEROL	IRRADIATED ANIMAL PROVITAMIN
Chick assay results in			
A.O.A.C. Units per gram			
Assay no. 1	157	389	468
Assay no. 2	225	425	500
Assay no. 3	160
Average	181	407	484
Poult assay results in			
" Poult units" per gram			
Assay no. 4	219	931	887
" Poult units"/A.O.A.C. Units	1.21	2.29	1.83

Each of the experiments with poults was of 4 weeks duration. The poults were weighed individually at weekly intervals and a record of feed consumption was kept. All poults were killed at 4 weeks of age, with the exception of group 1 which was terminated at 3 weeks, as noted in table 1. The left tibiae were removed, extracted, and ashed by groups at 850°C. according to the A.O.A.C. procedure.

RESULTS AND DISCUSSION

The average weights of the groups at 4 weeks of age, the efficiency of feed utilization, and the per cent bone ash are given in tables 1 and 2 for experiments 1 and 2, respectively. Mortality figures are not given, since mortality was negligible for all groups receiving vitamin D supplements. Excluding the basal group in experiment 1, of 168 poults started nine poults died. Excluding the basal group in experiment 2, of 128 poults started three died.

In the last column of table 1 and of table 2, the results of the bone ash determinations are interpreted in terms of "poult units" per gram of oil. For this purpose, a potency of 115 "poult units" per gram was assigned to the Reference Cod Liver Oil to correspond to its potency of 115 A.O.A.C. or U.S.P.XI Units per gram. For each experiment, per cent bone ash produced by each experimental mash containing Reference Cod Liver Oil was then plotted against the number of "poult units" of vitamin D supplied by that mash, and on the basis of the resulting points a straight line was drawn. The number of "poult units" corresponding to the per cent bone ash produced by each of the other mashes was then determined by the aid of these straight lines. The potencies thus calculated for the various levels of each of the vitamin D sources were then averaged and in table 3 these average figures are compared with the potencies in terms of A.O.A.C. chick units.

It will be noted that cod liver oil number 8-117 showed approximately the same potency whether tested with chicks or with poults, indicating that its vitamin D-active compounds were similar to those of Reference Cod Liver Oil in their comparative effectiveness for chicks and poults. This was not true of the two irradiation products. They contained approximately twice as many poult units as chick units, indicating that their vitamin D-active compounds were quite different from those of Reference Cod Liver Oil in their comparative effectiveness for chicks and poults.

These results confirm the findings of Boucher ('44), and together with those findings point to a difference between the vitamin D active compounds of the irradiation products on the one hand and those of fish oils on the other with respect to their comparative efficacies for chicks and poults. The figures presented in table 3 would seem to indicate a slight difference in this respect between the two irradiation products, but it does not appear that any significance should be attached to this difference since the biological assay method used is not very exact and since Boucher ('44) found a slight difference in the opposite direction.

The practice of evaluating vitamin D sources for turkeys on the basis of chick assays is certainly open to question, and any statement of the vitamin D requirement of poults in terms of A.O.A.C. Units requires some qualification. Considering the groups fed cod liver oil in these experiments, one would conclude that the poult's vitamin D requirement is about 200 A.O.A.C. Units per 100 gm. of feed. Considering only the groups fed the irradiation products, one would conclude that the poult's requirement is about 100 A.O.A.C. Units per 100 gm.

of feed. In neither case were sufficiently high levels fed to permit a really accurate statement of the "requirement," but the results clearly indicate the need for quotation marks around the word "requirement" when expressed in A.O.A.C. Units.

The results of these experiments do not supply an explanation of the specific disagreements existing in the literature concerning the poult's vitamin D requirement. The requirement determined in these experiments with Reference Cod Liver Oil agrees with the finding of Jukes and Sanford ('39) for Reference Cod Liver Oil and disagrees with the finding of Hammond ('41). The basal diet used by Hammond ('41) is calculated to contain 1.4% calcium and 0.8% phosphorus as compared with 1.06% calcium and 0.82% phosphorus in the diet used in these experiments. The higher calcium content and more favorable calcium: phosphorus ratio in Hammond's ('41) diet would be expected to result in a lower vitamin D requirement than would be the case with the diet used here. On the other hand the diet of Jukes and Sanford ('39) contained approximately 2% calcium and 1% phosphorus and hence should have required even less vitamin D than the diet of Hammond ('41). The diet of Carver and Rhian ('42) was very high in mineral, being calculated to contain about 2.4% calcium and 1.1% phosphorus. This fact as well as the possibility that their fortified cod liver oil contained activated animal sterol might explain the comparatively low requirement reported by them. The diet used by Baird and Greene ('35) was comparable with that of Hammond ('41) in mineral content. It contained 1.59% calcium and 0.87% phosphorus. Furthermore, the early date at which this work was done precludes the possibility that their fortified cod liver oil contained activated animal sterol.

It appears, then, that although at first glance the results of this investigation confirm the report of Jukes and Sanford ('39), these results can be reconciled with the reports of Baird and Greene ('35), Hammond ('41) and Carver and Rhian ('42) on the basis of calcium and phosphorus content of the diet whereas the report of Jukes and Sanford ('39) cannot. The use of Reference Cod Liver Oil by Jukes and Sanford ('39) and by Hammond ('41) excludes difference in vitamin D source from the list of possible explanations of the discrepancy between the reports of these investigators. Further work will no doubt be required to resolve this disagreement.

SUMMARY

Turkey poult's during the first 4 weeks of life were fed a vitamin D deficient diet supplemented with four levels of each of the following

vitamin D sources: U.S.P. Reference Cod Liver Oil no. 2, another unfortified cod liver oil, irradiated 7-dehydrocholesterol, and irradiated animal provitamin. On the basis of per cent bone ash, a given number of A.O.A.C. chick units of vitamin D from irradiated 7-dehydrocholesterol and irradiated animal provitamin were, respectively, 2.29 and 1.83 times as effective for poults as the same number of A.O.A.C. chick units from Reference Cod Liver Oil. The other sample of cod liver oil was similar to the Reference Oil in relative efficacy for poults and chicks.

• ACKNOWLEDGMENT

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SIMPLIFIED DIETS FOR THE GUINEA PIG^{1 2}

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Three groups of investigators have recently reported that the guinea pig requires dietary factors which have not been isolated and identified. Hogan and Hamilton ('42) found that guinea pigs would not grow or survive long on simplified diets in the absence of crude vitamin carriers. Dried yeast and a water extract of dried liver contain the unidentified essentials. Woolley ('42, '42 a) reported evidence that guinea pigs require three or more dietary essentials in addition to those required for the growth of rats and mice. Two of these factors occurred in linseed oil meal. An adequate source of the third was not found, but its existence was postulated to account for the fact that guinea pigs die after about 4 weeks on a highly purified diet supplemented with 25% linseed oil meal plus 2 to 5% dried grass (Ceroparyl). Sober, Mannering, Cannon, Elvehjem and Hart ('42) are also of the opinion that guinea pigs require three unknown dietary essentials. These factors are thought to be different from those required by the rat and the chick and are supplied by simultaneously supplementing a purified diet with yeast, dried grass and milk. Each of these supplements is presumably a good source of at least one of the three unknown factors.

Additional evidence concerning the unknown dietary essentials required by the guinea pig is presented in this paper, based upon data obtained since 1939. Preliminary experiments with approximately 600 animals served as a background for the work presented in this report. The study was planned as a progressive simplification of a satisfactory diet containing natural foods (diet G-31, a slight modification of the diet introduced by Sherman, La Mer, and Campbell ('22) for making vitamin C assays). In substituting for the natural foods, casein was used to supply protein, sucrose and rice starch to supply carbohydrates,

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²This paper is based upon a dissertation submitted to the Graduate School of the University of Pittsburgh by K. A. Kuiken in partial fulfillment of the requirements for the degree of Doctor of Philosophy, February, 1943.

rice cellulose to replace the crude fiber, and hydrogenated vegetable oil and corn oil to replace the fats; the salt mixture simulated the minerals of the original diet. All substitutions were based on analytical data given by Winton and Winton ('32, '37). The composition of the diets is given in table 1.

TABLE 1
Composition of diets.

CONSTITUENT	DIETS											
	G-27	G-28	G-29	G-30	G-31	G-32	G-33	G-34	G-35	G-39	G-40	G-41 ¹
Wheat bran	30.0		30.0	30.0	30.0		30.0					
Rolled oats	29.0	29.0		29.0	29.0	29.0						
Skim milk powder		30.0	30.0	30.0	30.0	30.0	30.0	30.0				
Sucrose	16.5								16.4	16.4	16.4	76.0
Rice starch		18.0	21.0			18.0	21.0	39.0	38.8	38.8	38.8	
Rice cellulose		2.5	0.5			2.5	0.5	3.0	3.0	3.0	3.0	
Casein (Borden's commercial)	10.7	5.2	5.0			5.2	5.0	10.2	21.0	21.0		
Casein (vitamin-free)											21.0	18.0
Butter fat	10.0	10.0	10.0		10.0							
Hydrogenated vegetable oil		1.9	2.1	10.0		11.9	12.1	14.0	14.0	12.0	12.0	
Corn oil										2.0	2.0	1.0
Dried grass												2.0
Salts ²	2.8	2.4	0.4			2.4	0.4	2.8	5.8	5.8	5.8	5.0
NaCl ³	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	

¹ Diet G-41 was prepared as described by Woolley ('42 a).

² Salt mixtures were planned to simulate the mineral content of the replaced foods. The final mixture contained 3.2 gm. $MgCO_3$, 2.3 gm. KCl , 8.4 gm. KH_2PO_4 , 3.2 gm. $Na_2HPO_4 \cdot 12H_2O$, 0.7 gm. $Ca_3(PO_4)_2$, 1.8 gm. $CaCO_3$, 0.04 gm. K_2CO_3 , 0.02 gm. $NaCl$, 0.06 gm. Na_2CO_3 , 0.6 gm. $NaH_2PO_4 \cdot H_2O$, and 0.5 gm. $CaHPO_4 \cdot 2H_2O$.

³ To each 100 gm. of iodized table salt were added 6 gm. iron and ammonium citrate (brown pearls), 0.2 gm. $CuSO_4 \cdot 5H_2O$, 0.26 gm. $MnSO_4 \cdot 4H_2O$ and 0.36 gm. $ZnSO_4 \cdot 7H_2O$.

Using a diet prepared in this manner it has been demonstrated that guinea pigs require an additional unknown dietary factor (or factors). When commercial casein provides the protein of the diet, small supplements of rice polish concentrate, brewers' yeast, liver extracts, dried grass, or skim milk powder permit survival and growth of guinea pigs over relatively long periods of time.

EXPERIMENTAL

Guinea pigs were purchased from a local dealer. Most of the work was done with male animals weighing from 200 to 300 gm.; both sexes were used in some of the experiments when sufficient males could not be obtained; younger animals were used during the latter part of the work. In each experiment the animals were distributed equally with regard to both weight and sex.

Individual cages with $\frac{1}{2}$ -inch mesh raised screen bottoms were used. Food and water were supplied ad libitum in open glass cups. All animals were weighed on alternate days. The adequacy of the diets was

TABLE 2
Oral supplements.¹

CONSTITUENTS	SUPPLEMENTS			
	E	G	I	K ²
Ascorbic acid, milligrams	5	5	5	5
Thiamine chloride, micrograms	50	50	50	150
Pyridoxine, micrograms	50	50	50	150
Riboflavin, micrograms	50	50	50	150
Nicotinic acid, micrograms	50	50	50	300
Inositol, micrograms	100	100	100	5,000
d-Calcium pantothenate, micrograms	200	200	200	400
Choline chloride, milligrams	5	5	5	15
Chondroitin, milligrams	5	5	5	5
Para-amino benzoic acid, milligrams		1	1	1
Calcium eriodictate, milligrams		2	2	2
β -alanine, milligrams			2	2
Pimelic acid, milligrams				1

¹ Synthetic vitamins were generously supplied by Merck and Company and Parke, Davis and Company.

² Animals which were fed water soluble supplement K daily, received about 1 mg. 2-methyl-1, 4-naphthoquinone weekly along with the vitamin A, D and E supplement.

judged on the basis of growth and survival over relatively long periods of time. Specific symptoms of deficiency were watched for constantly but were never observed with regularity in any one group.

The basal diets were prepared at approximately weekly intervals, and crude vitamin carriers were added not more than 24 to 48 hours before feeding. The feeding of "5%" of a supplement refers to mixing 5 gm. of the supplement and 100 gm. of the basal diet. Except in the case of diet G-41, supplements of known vitamins were not incorporated in the diet but were fed orally, by pipette. The oral supplements are listed in table 2. All animals received 5 mg. of ascorbic acid per day.

Vitamins A and D were supplied weekly at levels of 5,000 and 800 U.S.P. units, respectively, by feeding a mixture of fish liver oils³ and vitamin D₂.⁴ In the early experiments wheat germ oil was fed by pipette to supply vitamin E; synthetic alpha-tocopherol became available at a later date and was fed weekly at a level corresponding to 1 mg. per kilo body weight per day.

Some of the diets used in this study were very powdery as first prepared and guinea pigs refused to eat them in the powdered state. For this reason, such diets were mixed with a small amount of water, kneaded thoroughly and dried overnight in a low temperature oven (maximum 55°C.). The diets thus prepared were readily eaten by guinea pigs. A control experiment with a commercial ration indicated that this process did not alter the growth promoting properties of the diet.

RESULTS

Only representative data obtained during the last 3½ years are included in tables 3 and 4. These outline the approach to the problem and provide evidence that the guinea pig requires unknown dietary factors. Diets G-27 through G-34 were used in the progressive simplification of the natural food diet. The success with these led to the formulation of G-35 free of any of the natural foods of the original diet. G-39 differs from G-35 by containing 2% corn oil in place of an equivalent amount of hydrogenated vegetable oil. An 80-day period was selected for comparing various supplements to diets G-35 and G-39, although many of the experiments were continued for more than 100 days.

Guinea pigs do not grow appreciably when fed diet G-39 and a supplement of known vitamins plus various suggested nutritional factors. However, they survive for long periods of time upon such a ration. Seven of a group of ten animals lived for over 100 days on diet G-39 plus oral supplement G. Survival and growth were improved when the following crude vitamin carriers were fed as supplements to diets G-35 or G-39: rice polish concentrate,⁵ brewers' yeast,⁶ powdered liver extract,⁷ dried grass⁸ or skim milk powder. Of these individual supplements, rice polish concentrate was the richest source of the unknown factor required under the conditions described. An average growth

³ Natola, kindly supplied by Parke, Davis and Company.

⁴ Driadol, crystalline vitamin D₂ in propylene glycol, kindly supplied by the Winthrop Chemical Company, Inc.

⁵ Labco.

⁶ Pabst dried brewers' yeast.

⁷ Wilson's powdered liver extract 1-20 kindly supplied by Wilson Laboratories.

⁸ Generously supplied by the Cerophyl Laboratories.

rate of 2.2 gm. per day for an 80-day period was obtained when diet G-39 was supplemented with only 5% of this product.

When 10% of rice polish concentrate was fed along with 10% of commercial milk-vitamin-concentrate (diet G-35), growth was only equal to that obtained with rice polish concentrate at a 5% level. Combining

TABLE 3
Growth of guinea pigs on simplified diets.

RATION AND SUPPLEMENTS	ORAL SUPPLEMENT	NO. OF ANIMALS	TEST PERIOD	NO. OF SURVIVORS	AVERAGE GROWTH
			<i>days</i>		<i>gm./day</i>
G-27 + 5% M.V.C. ¹		16	100	9	3.7
G-28 + 5% M.V.C.		6	100	5	4.1
G-29 + 5% M.V.C.		6	100	6	4.0
G-30 + 5% M.V.C.		6	100	6	4.1
G-31 + 5% M.V.C.		5	100	4	4.4
G-32 + 5% M.V.C.		8	70	8	2.6
G-33 + 5% M.V.C.		8	120	6	2.7
G-34 + 5% M.V.C.		13	50	9	1.6
G-34 + 5% M.V.C. + 50 micrograms thiamine chloride per day		14	100	11 ⁷	2.8
G-35 + 10% M.V.C. + 10% R.P.C. ²		14	80	11 ⁸	2.1
G-39	G	10	80	7 ⁹	0.12
G-39 + 5% M.V.C.	E	14	80	8 ¹⁰	1.0
G-39 + 5% R.P.C.	E	19	80	17 ¹¹	2.2
G-39 + 5% R.P.C.	G	10	80	9	2.1
G-39 + 8% liver ³	G	5	50	4	2.1
G-39 + 6% grass ⁴	G	5	80	2	1.5
G-39 + 5% yeast ⁵	G	11	80	9	1.4
G-39 + 5% S.M.P. ⁶	G	11	80	9	2.2
G-39 + 5% R.P.C. + 8% liver	G	5	50	5	1.8
G-39 + 5% R.P.C. + 6% grass	G	5	80	5	3.0
G-39 + 5% R.P.C. + 5% yeast	G	11	80	10	2.6
G-39 + 5% S.M.P. + 5% yeast	G	8	80	8	2.7
G-39 + 5% R.P.C. + 5% S.M.P.	G	12	80	10	3.4

¹ M.V.C. = 70% ethanol extract of Borden's milk vitamin concentrate; 1 ml. = 1 gm. original product.

² R.P.C. = Labco Rice Polish Concentrate.

³ Powdered liver extract 1-20 from the Wilson Laboratories.

⁴ Dried grass from the Cerophyl Laboratories.

⁵ Pabst brewers' yeast.

⁶ S.M.P. = skim milk powder.

⁷ Eleven animals continued successfully for 210 days.

⁸ Seven animals continued successfully for 180 days.

⁹ Died after 110 to 150 days on experiment.

¹⁰ One lived 250 days; all others died during the first 120 days.

¹¹ Nine of an original group of 12 were continued and grew at an average rate of 1.6 gm. per day for a 190 day period.

rice polish concentrate with liver or yeast did not greatly improve the results obtained with rice polish alone, but further growth stimulation was observed when rice polish concentrate was combined with either skim milk powder or dried grass. An average growth rate of 3.4 gm. per day was obtained by supplementing diet G-39 with 5% of rice polish concentrate plus 5% of skim milk powder and oral supplement G.

Representative results obtained with basal diets of higher purity (G-40 and G-41) are presented in table 4. Diet G-40 was prepared by replacing the commercial casein of diet G-39 with "vitamin-free"

TABLE 4
Experiments with diets containing vitamin-free casein.

RATION AND SUPPLEMENTS ¹	NO. OF ANIMALS	NO. OF SURVIVORS			AVERAGE GROWTH (AT 20 DAYS)
		20 days	30 days	40 days	
G-40 (S.M.A. casein) + oral supplement G	20	10	3	2	gm./day — 3.1
G-40 (Labco casein) + oral supplement G	20	10	3	2	— 2.6
G-40 + 10% S.M.P. + oral supplement G	10	8	4	4	— 2.5
G-40 + 10% R.P.C. + oral supplement G	10	7	2	0	— 2.6
G-40 + 5% R.P.C. + 5% S.M.P. + oral supplement G	10	5	4	2	— 1.0
G-40 + 5% R.P.C. + 10% grass + 1% glycine + oral supplement I	6 ²	5	4	0	0.0
G-40 + 5% R.P.C. + 5% S.M.P. + 10% grass + 1% glycine + oral supplement I	7 ²	7	6	0	0.10
G-40 + 10% liver + 10% grass + oral supplement I	11 ²	4	0	0	
G-40 + 10% yeast + 10% grass + oral supplement K	34 ²	16	6	5	0.52
G-41 (Basal controls)	10 ²	3	2	1	0.17
G-41 + oral supplement K	10 ²	6	4	3	0.50
G-41 + 25% linseed oil meal	10 ²	6	2	2	0.50
G-41 + 10% R.P.C.	10 ²	10	8	6	1.4 ³
G-41 + 10% S.M.P.	10 ²	7	6	4	0.07
G-41 + 10% yeast	10 ²	8	8	7	1.3 ⁴
G-41 + 10% liver	10 ²	10	10	10	0.75 ⁵
G-41 + 10% R.P.C. + 10% S.M.P.	10 ²	10	9	8	1.0 ⁶
G-41 + 10% R.P.C. + 10% M.V.C. + oral supplement K	10 ²	10	7	4	1.2

¹ Refer to table 3 for explanation of abbreviations and identification of supplements. Unless otherwise indicated, Labco Vitamin-free casein was used in preparation of the diets.

² Starting weights ranged from 125 to 200 gm. All others ranged from 200 to 300 gm.

³ Survivors averaged 1.4 gm./day for 40 days.

⁴ Survivors averaged 0.36 gm./day for 40 days.

⁵ Survivors averaged 0.64 gm./day for 40 days.

⁶ Survivors averaged 1.0 gm./day for 40 days.

casein. Guinea pigs did not survive on diet G-40 in contrast with their performance on diet G-39. The crude vitamin carriers which were effective in earlier experiments only permitted slight improvement in the survival and growth of guinea pigs in comparison with their performance on the basal ration alone. The crude vitamin carriers which were markedly effective in earlier experiments only produced slight improvement in the survival and growth of guinea pigs in comparison with their performance on the basal ration alone. The commercial casein (21%) of diet G-39, therefore, either supplied a basal level of some necessary factor which was increased to more nearly an adequate level by the various supplements or it carried an essential material different from those provided in appreciable dosage in the supplements. If only one essential were involved, the large additions to the vitamin-free casein diet should at least have permitted survival comparable with that on the unsupplemented commercial casein diet. Of the 129 animals on diet G-40 plus supplements (of 10% at least, and half the group received a 20% supplement), 91 animals survived 20 days, only 12 survived 40 days and none lived beyond 50 days. In contrast, on diet G-39 without any supplement, 7 of a group of 10 survived 110 days or longer, and on G-39 plus a 5 or 10% supplement, 96 of 116 animals survived 80 days (end of experimental period) and grew fairly satisfactorily. These data may not exclude the possibility of explaining the differences in performance on the basis of different levels of the same factor, but they argue very strongly for two different factors — one supplied by the commercial casein and another by the various supplements. The authors are fully convinced that the data justify the latter interpretation.

Diet G-41, used successfully by Woolley ('42, '42 a) in assays with very young animals, also was used to test the above group of crude vitamin supplements. Guinea pigs ate this diet more readily from the start than diet G-40 and, accordingly, growth stimulation over a short period of time could be more easily demonstrated. Rice polish concentrate increased growth more uniformly than other supplements. Liver extract was very effective in extending the average survival time but did not have an equal effect on growth. The one sample of linseed oil meal tested did not markedly improve either growth or survival. However, the results should not be compared directly with Woolley's observations because our animals were not as young as those he used. A complete diet was not produced in any of the combinations, in the sense of maintaining *normal* growth. Survival of guinea pigs on the highly purified diet was barely extended beyond 40 days.

DISCUSSION

Guinea pigs survived and grew although at a restricted rate, for long periods of time on simplified diets containing not more than 5% of crude vitamin concentrates. One group of nine animals was discontinued after 190 days with an average growth of 1.6 gm. per day. The successful diets can hardly be referred to as "purified diets", however, because they did not consist solely of highly purified ingredients. When an attempt was made to use "vitamin-free" casein in the preparation of the diet, guinea pigs failed to grow or survive satisfactorily. Commercial casein (Borden), therefore, contains a generous supply of a dietary factor essential for the guinea pig, beyond those required by rats, that is not present in "vitamin-free" casein.

It was not possible to characterize the limiting factors involved when the study was extended with diets containing purified casein. Although biotin and "folic acid" were not available for testing, it is doubtful whether a deficiency of either of these factors was involved. Cow's milk has been reported to be extremely low in "folic acid" (Cheldelin and Williams, '42), and casein or skim milk powder therefore, would not be expected to carry it in large enough quantities to account for the results. On the other hand, milk is a good source of biotin (Cheldelin and Williams, '42; and György, '39) and the change in casein might have critically reduced the intake of this factor. However, yeast and fresh liver have been reported to be good sources of biotin (György, '39), and it will be noted that growth stimulation was not observed when 10% of either dried brewers' yeast or liver extract was added to diet G-40 plus 10% of grass. Since no direct analytical data are available on the yeast and liver preparations tested, though they were standard high quality products, it can only be assumed that they were contributing significant amounts of biotin. The rice polish concentrate used in this work contained only 1.4 $\mu\text{g.}^9$ of biotin per gm. of solids and, therefore, cannot be regarded as a very potent source of this factor. This indirect evidence suggests that neither biotin nor "folic acid" is the factor supplied by commercial casein.

Sober, Mannering, Cannon, Elvehjem and Hart ('42) have reported that the general condition of their guinea pigs was improved when the amount of casein in the basal ration was raised from 18% to 30%. Since the casein used in their ration was "relatively unpurified" the beneficial effect observed may be related to an increased supply of the unknown dietary essential reported here. Our data suggest that this factor precipitates with the casein fraction during the commercial

* Data kindly supplied by G. C. Supplee of Borden Company Research Division.

preparation of casein, since a 10% skim milk powder supplement did not permit a detectable growth response in animals receiving a "vitamin-free" casein diet even though it may have improved the survival slightly.

Animals showed a relatively good survival when fed the basal diet (containing commercial casein plus oral supplement G), but they failed to grow satisfactorily. Only a few crude vitamin carriers were tested as supplements to this diet. Rice polish concentrate was consistently found to improve the diet and is regarded as the best source (of those studied) of at least one unknown factor required by the guinea pig. Brewers' yeast, powdered liver extract, skim milk powder, and dried grass were also found to be valuable as supplements, and it is thought that they contain lesser amounts of the same factor (or factors) supplied by the rice polish concentrate.

The beneficial effect of the commercial casein and also of the skim milk powder is in accord with the good growth response obtained by Sober, Mannering, Cannon, Elvehjem, and Hart ('42) to supplements of 20 ml. of milk per day fed to guinea pigs on a simplified ration. However, in contrast with the diets used in our experiments, their diets contained both yeast and dried grass, and their supplements of known vitamins were more limited both qualitatively and quantitatively. Our oral supplements (table 2) included not only liberal quantities of known vitamins, but also a number of materials which have been suggested as essential nutrients. The necessary factors supplied by the yeast and grass may have been included in part, in the oral supplements fed in the present experiments. The liberal provision of our vitamin supplement is thought to furnish good evidence that the factors reported here are different from any of the well characterized nutritional essentials. The chance that an amino acid deficiency might be involved seems very remote because of the very limited responses obtainable from the milk, yeast and dried grass supplement.

It is of interest to note that albino rats, initial weight 50 gm., grew well on diet G-39 plus 5% of the rice polish concentrate and supplement E (at one-half the guinea pig level) through a period of 50 days (average gain in body weight, 3.6 gm. per day).

"Wrist stiffness" in the guinea pig as reported by Wulzen and Bahrs ('41) and Wulzen ('42) was not observed; only nine cases of stomach ulceration (Kohler, Randle, Elvehjem and Hart, '39) were found and these were not observed to be associated with any specific variant in the diets. It must be concluded that if these are specific deficiency symptoms, the corrective factors were present in the diets studied either directly or by intestinal synthesis.

SUMMARY

1. Growth and survival of guinea pigs for long periods of time on simplified diets containing not more than 5% of crude vitamin concentrates are reported.

2. The basal simplified diet was developed through systematic replacement of the natural foods in a satisfactory ration. With equivalent amounts of protein, carbohydrate, fat, and minerals, the diet was not adequate when supplemented with known vitamins.

3. The authors interpret the data presented as indicating that at least two unknown dietary essentials are required to complete the diet of the guinea pig. When commercial casein provides the protein of the diet, small supplements of rice polish concentrate, brewers' yeast, liver extract, skim milk powder or dried grass markedly improve both survival and growth, thus demonstrating the presence of one of these essentials. However, if "vitamin-free" casein is used, these supplements, even in large amounts, have very little, if any, beneficial effect upon growth or survival, providing evidence for another factor in commercial casein.

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STUDIES ON THE ABSORPTION OF CAROTENE¹

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ONE FIGURE

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Because of the increasing importance of the provitamin, carotene, as a source of vitamin A in the diet, it is desirable to have information on the factors which influence the absorption and utilization of this pigment from the gastrointestinal tract. There is considerable evidence that the bile plays an essential rôle in the absorption of carotene. Greaves and Schmidt ('35) proved that the bile salts such as glycodesoxycholic or desoxycholic acid were required for the absorption of this provitamin in vitamin A deficient, choledochocolonostomized rats as judged by the vaginal smear technique. Vitamin A on the other hand could be readily absorbed by such bile fistula rats. Similar convincing results on dogs have been reported by Irvin, Kopala and Johnston ('41) making use of intestinal loops; insignificant amounts of carotene were absorbed when introduced into the loops in cotton-seed oil solutions; however, when either gall-bladder bile (hog or ox) or pancreatic lipase was administered, significant quantities of carotene were absorbed. When both lipase and bile were introduced together with the carotene solution, much larger amounts were utilized. Sodium desoxycholate was considerably more effective than the sodium salts of cholic or glycocholic acid and somewhat more potent than whole bile. The differences in absorption of carotene and vitamin A have been ascribed to the fact that the latter is an alcohol while the former is only hydrocarbon.

Carotene absorption is apparently augmented when fat is also being absorbed. Wilson, Das Gupta and Ahmad ('37) found practically complete absorption of the carotene of raw or cooked carrots and of spinach when the diet contained fat while only 50% was absorbed on a fat-free diet. Van Eekelen and Pannevis ('38) obtained similar results with human subjects while Kemmerer and Fraps ('38) found in rats

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increased absorption of carotene when fed in Wesson oil. Russell, Taylor, Walker and Polskin ('42) report that the absorption of carotene but not of vitamin A was decreased in hens on a fat-free ration. The percentage absorption of carotene was also higher when fed in lower concentrations. It is also possible that the absorption of this pigment may be different when fed in the pure state than when fed as a component of foods.

The absorption of vitamin A by normal rats has been shown by Reifman, Hallman and Deuel ('43) to vary with concentration. Although the rate of absorption of vitamin A was only approximately 6 I.U. per 100 sq. cm. per hour when administered in fat in a concentration of 100 I.U. per gram, it was increased to over 10,000 I.U. when the concentrate contained approximately 1,000,000 I.U. per gram.

The present study was designed to determine the rate of absorption of carotene as influenced by concentration. It has been possible not only to determine the period necessary for absorption from the lumen of the gut but also to follow the transfer from the gut wall to the tissues. Experiments are now in progress to determine when factors may influence each of these steps.

EXPERIMENTAL

Carotene dissolved or suspended in cotton-seed oil was fed by stomach tube to rats previously fasted for 48 hours and the amount of carotene remaining in the gut determined after various periods. It was necessary to place the rats which had been on the stock diet high in alfalfa, on a carotene-low diet² for a week prior to the fasting period in order to decrease the correction for pigment in the intestine.

Carotene was determined by the procedure of Koehn and Sherman ('40) with the Klett-Summerson photoelectric colorimeter and a filter with a maximum transmission at 420 m μ . Total fat was determined on the gastro-intestinal contents as described previously (Deuel, Hallman, and Leonard, '40). In the experiments reported in series I, carotene was determined only on the lumen contents. In the second series of tests, after removal of the contents of the lumen by flushing with 100 ml. of freshly distilled diethyl ether, the carotene was also determined in the gut wall by extraction of the gut homogenized in the Waring blender three times with diethyl ether. The extracts from the lumen contents were dried with anhydrous Na₂SO₄, the extract filtered and made to volume, the Na₂SO₄ being thoroughly washed with ether. Caro-

² Similar to U.S.P. XII vitamin A depletion diet except that unextracted casein was used.

tene was determined on this sample and the total lipid was estimated on an aliquot by evaporation of the diethyl ether.

With the extracts of the intestinal wall, the extract was filtered, made to volume with diethyl ether, and the value of carotene read directly on the colorimeter. Carotene was administered in amounts of approximately 350 and 3750 $\mu\text{g.}$ per ml. of fat. In the former, all the carotene was in solution; in the higher concentration, 35.2% or 1322 $\mu\text{g.}$ was in solution and 64.8% or 2428 $\mu\text{g.}$ was in suspension and could be separated by centrifugation at 10,000 r.p.m. for 1 hour. The mixtures were fed in amounts of 300 mg. per 100 sq. cm. of surface area. Surface area was computed by the formula of Lee ('29) based on the weight after fasting. The carotene was the S.M.A. product containing 90% beta and 10% alpha carotene.

TABLE 1

The carotene and lipid recovered from the gastro-intestinal contents of male rats previously fasted for 2 days (control tests) or immediately after the administration of carotene in cottonseed oil (recovery tests).

NO. OF TESTS	AVERAGE WEIGHT	LIPID				CAROTENE				
		Fed	Recovered			Fed	Recovered			In gut wall
			Total	Corrected	Per cent		Total	Corrected	Per cent	
Control tests		mg.	mg.	mg.		μg.	μg.	μg.		μg.
7	253	0.0	14.4			0.0	9			
3	218	0.0	13.9			0.0	1			
6	256	0.0				0.0	6			69.9
Recovery tests										
7	274	1001	1002	988	98.9	363	359	350	95.4	
5	222	888	873	859	97.0	3693	3572		96.5	

RESULTS

Table 1 records the control values of carotene and lipid in the intestinal contents and intestinal wall of fasted rats, values which are used as correction factors as well as in the estimation of the recovery of these constituents when the gut was removed and flushed with ether immediately after the carotene solutions were introduced.

Table 2 gives the summary of the average absorption at 3 hours when the carotene was fed at different concentrations (series I) and for the larger doses at various intervals for 42 hours (series II). The total recovered is the sum of that found in the lumen and gastro-intestinal wall. The total transferred refers to the quantity which is unaccounted for, presumably because it was passed through the intestinal mucosa

TABLE 2

The absorption of lipid and carotene by rats fed 300 mg. per 100 sq. cm. of carotene solutions in cottonseed oil and killed at various periods thereafter.

LENGTH PERIOD	BODY WT.	LIPID				CAROTENE					
		Fed	Absorbed *		Fed	Recovered *			Transferred *		
			Total	Per 100 sq. cm./hour		Total	Lumen	Wall	Total	Per 100 sq. cm./hour	Absorbed *
Series I	Ar.	gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
3	257	963	348±27 (144-535)	31.0±2.4 (15.7-49.1)	347	253±9 (195-316)					
3	236	914	325±24 (218-449)	32.9±1.6 (31.4-45.6)	3806	2734±95 (1800-3500)					
Series II	3	242	931	34.2±2.5 (21.7-49.5)	3821	2681±110 (2237-3286)	711±97 (270-1208)	429±115 (74-1289)	52.6±11.2 (4.8-128.7)	1140±108 (606-1675)	112.0±10.4 (58.5-160.5)
6	231	904	632±33 (469-872)	32.4±1.9 (22.6-42.5)	3689	1912±235 (957-3025)	875±136 (305-1750)	902±205 (11-2064)	48.0±9.2 (0.6-106.7)	1774±222 (651-2611)	90.7±11.3 (33.7-137.5)
12	225	890	858	22.2	3641	481±111 (80-1200)	2040±269 (260-3250)	1120±244 (499-3244)	29.1±8.1 (12.0-84.3)	3110±130 (2618-3940)	85.3±4.0 (64.3-113.5)
18	231	906	894	15.1	3698	362±135 (87-1565)	1778±226 (558-3070)	1558±218 (401-2699)	26.3±3.6 (7.1-43.7)	3330±182 (1973-4017)	56.1±2.4 (34.8-61.2)
42	222	880	880		3805	49±25 (0-232)	578±176 (0-1870)	2978±228 (1775-3647)	22.1±1.4 (12.3-26.8)	3567±33 (3378-3753)	

Male rats were used in series I, female rats in series II.

* 14 experiments in this group only; 10 experiments in the other groups.

* Including the standard error of the mean calculated as follows: $S.E.M. \sqrt{\frac{ed^2}{n}} / \sqrt{n}$ where "d" is the deviation and "n" is the number of observations. The values in parentheses indicate the maximum and minimum in each group.

* The "rank order" coefficient shows the direct relation between rate of lipid and carotene absorption. A perfect correlation would give a value of 1.00, while a value of 0.0 would indicate no correlation. The following formula was used for its calculation $\rho = 1 - \frac{6 \sum d^2}{n(n^2-1)}$ where "d" is the difference between the rank order and "n" is the number of determinations.

into the circulation. The quantity absorbed refers only to the amount which has disappeared from the lumen. This is the basis on which the absorption of fats and vitamin A has been computed in our previous work.

A comparison of the rate of absorption of both carotene and vitamin A with the concentration fed is given in figure 1. When this is based on I.U., the values for carotene at the two levels fed fall on the vitamin curve.

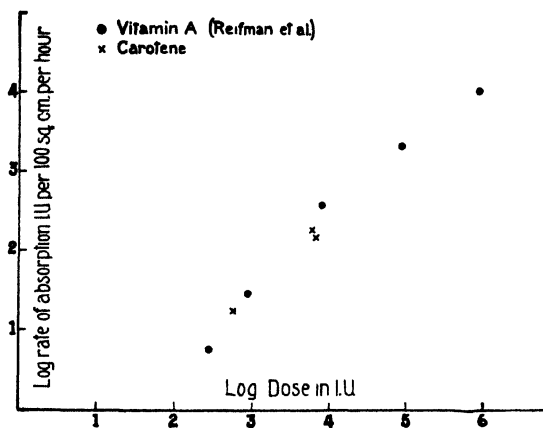


Figure 1

DISCUSSION

With a ten-fold increase in the concentration of the carotene fed, a proportionate increase in the quantity of carotene absorbed resulted. Thus, when approximately 350 $\mu\text{g.}$ of carotene were administered, the average absorption was 9 $\mu\text{g.}$ per 100 sq. cm. per hour. When the carotene administered was increased to 3800 $\mu\text{g.}$, the average rate of absorption in the two series of 3-hour tests was 108 and 112 $\mu\text{g.}$ The absorption rate dropped as the concentration in the lumen became lower, the calculated values being 91, 85 and 56 in the 6-, 12- and 18-hour tests, respectively. When the absorption levels in the 3-hour tests are compared with the values obtained earlier for vitamin A by Reifman, Hallman, and Deuel, '43, (fig. 1), the rates of absorption correspond closely to those obtained when the similar concentrations of vitamin A were given. This is especially remarkable in view of the fact that over 60% of the carotene in the higher dose was merely suspended in the oil.

As has been indicated by a number of investigators, there is a distinct correlation between the rate of carotene and lipid absorptions. This

interrelationship was found only in the 3- and 6-hour groups; no relationship could be expected in the 12-hour group since in most cases all of the lipid had been completely absorbed.

Carotene absorption was more rapid and complete than was to be expected; less than 2% remained in the gut 42 hours after feeding. The completeness of the absorption is difficult to understand when one considers that values of only 50% were obtained by Kemmerer and Fraps ('38) when carotene was fed in Wesson oil. Although we did not analyze the feces, when any excretion occurred it was usually in the early part of the experiment before the fat had reached the large intestine. All experiments in which diarrhea occurred were discarded. The discrepancy between the complete absorption in our tests and the less complete absorption obtained by other investigators may be that in the present tests the rats were fasted while in other tests the animals were fed simultaneously with the absorption experiment. It is possible that the presence of indigestible food residues in the latter cases may have resulted in the removal of undigested carotene from the intestine before absorption could be completed since the rate of absorption is quite slow.

The accumulation of carotene in the gut wall reached a maximum in the 12-hour group at which time the intestine grossly appeared colored deeply yellow. Since the rate of disappearance for the first 12 hours is less than the rate of accumulation in the gut wall, it is evident that the rate of transfer out of the gut wall may be a controlling factor in absorption of this pigment. The maximum average rate of removal from the lumen of 112 $\mu\text{g.}$ per 100 sq. cm. involves an absorption of 1150 $\mu\text{g.}$ or 1920 I.U. of carotene in a 3-hour period.

The rate of absorption of the cottonseed oil was quite uniform in the 3- and 6-hour tests (31.0 and 34.2 mg. per 100 sq. cm. per hour) which is somewhat lower than the value 43.3 obtained on female rats or the values 39.7–47.9 obtained on male rats reported by Deuel, Hallman, and Leonard, '40.

SUMMARY

The rate of absorption of carotene is proportional to the dose fed. When fed in cottonseed oil at a level of 350 $\mu\text{g.}$ per gram, 9 $\mu\text{g.}$ were absorbed per 100 sq. cm. per hour; at a level of 3750 $\mu\text{g.}$, 110 $\mu\text{g.}$ were absorbed per hour. These values calculated as I.U. are practically identical with those reported earlier for vitamin A. This occurred in spite of the fact that about two-thirds of the carotene fed in the higher dose was in suspension.

The concentration reaches a maximum in the gut wall at 12 hours; considerable amounts still remained in the gut wall after 42 hours, but all had been removed from the lumen of the gut. It is suggested that in the absorption of carotene the transfer through the intestinal wall is the limiting factor since it proceeds more slowly than absorption from the lumen. There is considerable correlation between the amounts of fat and carotene absorbed.

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EFFICACY OF VITAMIN D FROM DIFFERENT SOURCES FOR TURKEYS¹

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TWO FIGURES

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It is well known that turkeys have a higher requirement for certain specific nutrients than chickens and this is particularly true of vitamin D as first pointed out by Scott, Hughes and Loy ('32). Since then, however, there has been considerable disagreement among those who have attempted to measure the amount of vitamin D required by young growing turkeys. Baird and Greene ('35) using a single vitamin D supplement, a fortified cod liver oil, determined the levels required to prevent rachitic symptoms in growing chicks, poults and pheasants. They reported that turkeys require a minimum of 60 to 70 U.S.P. units of vitamin D per 100 gm. of feed during the first 12 weeks of life. A careful study of this work indicated that these authors may not have obtained the maximum response in calcification and growth from even the highest level of vitamin D fed in their turkey experiments and that, therefore, the requirement for maximum response could have been distinctly higher. Furthermore, the potency of their supplement was originally established in Steenbock rat units and the question of the conversion factor used in calculating U.S.P. units would also tend to raise doubt as to the true value of the units fed by them as compared with A.O.A.C. chick units used by later workers.

Jukes and Sanford ('39) were the first to attempt the quantitative determination of the response of young turkeys on a rickets-producing diet to a standard vitamin D substance, namely, U.S.P. reference cod liver oil no. 1, the potency of which was definitely known in terms of A.O.A.C. chick units. They also fed in the same experiments a sardine oil, fortified with fish liver oils, which was assayed with chicks against

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the reference cod liver oil. They found that levels of the reference cod liver oil up to and including 120 A.O.A.C. units per 100 gm. of diet produced unsatisfactory growth and calcification to 4 weeks of age. Unfortunately, they did not feed higher levels but extrapolation of their curve of response indicated that more than 200 units would be needed for maximum calcification. Surprisingly, they noted that levels of the fortified sardine oil, equivalent to the reference oil in A.O.A.C. chick units, produced a greater calcifying effect in the turkeys. This led them to suggest the possibility of a difference between chicks and turkeys in their response to different sources of vitamin D and they properly pointed out the difficulty of expressing the requirements of turkeys in terms of chick units.

Hammond ('41) in a brief paper reported the feeding of reference cod liver oil, a fortified cod liver oil and activated animal provitamin D to poults during the first 4 weeks of life as supplements to a rachitogenic diet. In sharp contrast with the findings of Jukes and Sanford ('39) he found that 80 A.O.A.C. chick units from the reference cod liver oil produced a maximum effect on body weight and bone calcification. His data on the other two supplements are of less value in estimating the efficacy of each since he relied on the manufacturer's claims for the A.O.A.C. chick unit potency. In addition, he presented no information on the source of the vitamin D used to fortify the cod liver oil, and the levels fed of activated animal provitamin D, except one, were unreasonably high. It is interesting to note, however, that the 50-unit level of activated animal provitamin D gave a response approximately equal to 80 units of the other two supplements.

After the work here reported had been started, the publication of Carver and Rhian ('42) appeared. These workers fed a fortified cod liver oil at 8 levels (50 to 200 units) and activated animal provitamin D at 1 level (100 units) to duplicate groups of poults receiving a rachitogenic diet. Both supplements were assayed by the authors and the dosage was expressed in A.O.A.C. chick units. No reference cod liver oil was fed. Carver and Rhian concluded that 80 A.O.A.C. chick units per 100 gm. of feed from either of the supplements produced satisfactory growth and calcification in young turkeys. No information was given as to the source or composition of the fortified oil.

The primary object of the experiments here described was to compare the efficacy of vitamin D from different sources for turkeys, particularly when fed on the basis of equal potency, measured in A.O.A.C. chick units. It appeared also that the observations of Jukes and Sanford ('39) were worthy of further investigation and that additional

work of this nature might help resolve the lack of agreement as to the requirement of young turkeys for this essential nutrient.

EXPERIMENTAL

The percentage composition and analysis of the basal diet are given in table 1. This diet was formulated with the idea of supplying optimum amounts of all the essential nutrients with the exception of vitamin D. It resembled typical turkey starting mashers except that casein was used in place of meat scraps or fish meal in order to avoid possible contamination with vitamin D from animal sources. The dehydrated alfalfa meal was of high quality as shown by analyses indicating a carotene content equivalent to 92,400 U.S.P. units of vitamin A per pound

TABLE 1
Percentage composition and analysis of basal diet.

Ground yellow corn	25.9	Ground oats	10.0	Dried brewers' yeast	6.0
Soybean oil meal	12.0	Dry skimmilk	8.0	Ground limestone	2.0
Wheat bran	10.0	Dehydrated alfalfa		Corn oil (Mazola)	2.0
Wheat middlings	10.0	meal	7.5	Sodium chloride	0.5
		Commercial casein	6.0	Manganese sulfate	0.1
Protein (N x 6.25) %	24.56	Crude fiber %			5.18
Moisture %	9.74	Calcium %			1.05
Ash %	5.46	Phosphorus %			0.63
Ether extract %	5.43	Manganese %			0.01
Riboflavin — micrograms per 100 gm.					575
Vitamin A — International units per 100 gm.					1724

and a riboflavin content of 5,630 µg. per pound. A vitamin D assay of the alfalfa meal using rats as experimental animals showed that it contained between 363 and 454 U.S.P. units per pound. This amount was considered negligible since it was presumed to be the ineffective activated plant sterol type. The results proved this to be true.

A preliminary trial employing 3 groups of poults was conducted for the purpose of testing the suitability of the basal diet described in table 1 and of securing some indication of the response which might be obtained to two supplements representing diverse sources of vitamin D. One group of poults received the basal diet alone, the second received as a supplement to the basal 115 A.O.A.C. units per 100 gm. of diet from fortified sardine oil, while the third received as a supplement 105 A.O.A.C. units per 100 gm. of diet from irradiated 7-dehydrocholesterol. The groups were continued for 4 weeks on these diets. On the twenty-eighth day the survivors were killed and bone ash determined by the

method described below. Of the ten poults started on the basal, only three survived; these had an average bone ash of 30.3%. Nine poults that received the sardine oil had an average bone ash of 45.1% while the eight poults that received the irradiated 7-dehydrocholesterol had an average bone ash of 49.0%. There was no mortality in the two groups of poults to which supplements were given.

These preliminary results indicated that the basal diet was satisfactory for producing rickets and that there might well exist distinct differences between different vitamins D in their ability to prevent rachitic manifestations in poults. Thus encouraged, a more extensive and quantitative comparison of four vitamin D supplements from different origins was undertaken. Two separate experiments were carried out in which these supplements were fed at various levels, experiment 2 being planned and carried out after the results of experiment 1 had been carefully studied.

The vitamin D supplements employed were: (1) U.S.P. reference cod liver oil no. 2; (2) a corn oil solution of an irradiated animal sterol; (3) a corn oil solution of irradiated 7-dehydrocholesterol; and (4) a fortified sardine oil.² These materials were chosen for the reason that they were representative of vitamins D of diverse origin and also of those largely used in modern poultry feeds. The reference cod liver oil was a standard test substance of known vitamin D content and in addition was presumed to be representative of cod liver oils in general. The fortified sardine oil was supplied with the assurance that all of its vitamin D activity came from fish oil sources. It was assumed that its vitamin D activity would be representative of sardine oil plus whatever high potency liver oil was used for increasing its D content. The irradiated animal sterol was prepared by irradiating with ultraviolet light the purified sterol mixture isolated from a species of mussel. The exact chemical nature of the vitamin D which it contained has not been reported. The irradiated 7-dehydrocholesterol, on the other hand, contained a single known vitamin D, namely D₃. Both irradiated products have come into wide commercial use under the designation of D-Activated Animal Sterol.

The various levels of the supplements were introduced into the diet by pre-mixing with the corn oil component. In order to establish their potency with accuracy the irradiated animal sterol, the irradiated 7-

² Thanks are expressed to E. Fullerton Cook, Chairman of the Committee of Revision of the Pharmacopoeia of the United States of America for the reference cod liver oil, to J. Waddell of E. I. du Pont de Nemours and Company for both the irradiated animal sterol and irradiated 7-dehydrocholesterol, and to T. D. Sanford of F. E. Booth Company for the fortified sardine oil.

dehydrocholesterol, and the fortified sardine oil were carefully assayed by the A.O.A.C. chick method in three separate laboratories. The results of the separate assays and the average value assigned to each supplement are summarized in table 2. It is believed that the assigned values have a high degree of accuracy.

White Holland and Bronze turkey poults were used in these investigations, representatives of both breeds being included in each group. Twelve to fourteen poults in each group were started when 1 day old and kept for 28 days in electrically heated battery brooders equipped with hardware cloth floors and automatic waterers. The brooders were

TABLE 2
A.O.A.C. chick assays of the vitamin D concentrates.

LABORATORY ¹	IRRADIATED ANIMAL STEROL	IRRADIATED 7-DEHYDROCHOLESTEROL	FORTIFIED SARDINE OIL
	<i>Units per gm.</i>	<i>Units per gm.</i>	<i>Units per gm.</i>
1	445 (4) ²	340 (4)	435 (4)
2	404 (4) 403 (3)	342 (4)	408 (4)
3	423 (3) 399 (3)	415 (3) 411 (3)	450 (3)
Assigned values	415	375	430

¹ Laboratory 1, the Department of Agricultural and Biological Chemistry of The Pennsylvania State College. Laboratory 2, the Laboratory of Vitamin Technology, Chicago. Laboratory 3, the Biological Laboratory of E. I. du Pont de Nemours and Company, New Brunswick, N. J.

² The figure in parentheses following each assay value represents the number of groups of chicks used in that assay.

located in a laboratory from which all direct sunlight was excluded by covering the closed windows with heavy brown paper. The experimental diets and tap water were supplied ad libitum.

At the conclusion of each experiment the surviving poults were killed and the tibias removed for ash determination. This was done essentially as outlined in Official and Tentative Methods of Analysis ('40), except that the extraction was continued for 30 hours with 95% ethyl alcohol followed by 30 hours with ethyl ether. The extracted bones were dried to constant weight at 103°C. and then ashed by placing the crucibles in a cold furnace and raising the temperature slowly to 400°C. The total time required for this method of ashing approximated 16 hours, during the last three of which the temperature was increased to 800°C. While the bones were individually ashed only the

average value for each group has been recorded in the subsequent tables.

RESULTS AND DISCUSSION

In the first experiment nineteen groups of poult were used. These were divided as follows: poult in one group received the basal diet alone; six groups of poult received the basal plus levels of reference cod liver oil ranging from 20 to 200 units per 100 gm. of diet; and four groups of poult were placed on each of the other three supplements. The levels at which these supplements were fed were chosen in the hope that they would yield responses which could be compared quantitatively with those given by the reference oil. The range originally planned was from 20 to 120 A.O.A.C. units of each supplement per 100 gm. of diet but the final assigned A.O.A.C. potency, calculated after the turkey experiments had been started, modified these values slightly. Detailed information on the results obtained from experiment 1, such as mortality, body weight, dry weight of bone, and bone ash percentage is summarized in table 3.

TABLE 3
Summary of experiment 1.

GROUP	SUPPLEMENT PER 100 GM. BASAL DIET			MOR- TALITY DUE TO RICKETS %	RESULTS AT 4 WEEKS OF AGE			
	Source	mg.	A.O.A.C. units		No. of poult	Body weight gm.	Tibia weight gm.	Tibia ash %
1	None	0	0	92	1	112	.4221	25.5
2	Reference	174	20	92	1	192	.5780	32.4
3	cod liver	348	40	67	4	209	.6776	32.8
4	oil	696	80	0	12	260	.8352	35.0
5		1043	120	0	12	307	.9915	42.7
6		1391	160	0	12	285	.9199	42.2
7		1739	200	0	13	302	.9780	44.6
8	Irradiated	47	19	42	7	176	.5885	31.5
9	animal	93	39	9	10	247	.7770	37.4
10	sterol	186	77	0	13	265	.8069	47.7
11		279	116	0	12	276	.8703	48.7
12	Irradiated	47	17	64	4	186	.5845	31.0
13	7-dehydro-	93	35	8	11	217	.7302	34.8
14	cholesterol	186	70	0	13	335	1.0677	45.8
15		279	105	0	13	299	.9304	46.1
16	Fortified	44	19	69	4	187	.5648	27.4
17	sardine	89	38	42	7	210	.6933	31.3
18	oil	178	76	22	7	242	.7442	38.3
19		267	115	0	12	294	.9261	41.2

In order to confirm the surprising differences in efficacy noted among the supplements in the first experiment, a second was carried out. In this second experiment 20 groups of poultts were used, these being distributed as in the first except that 7 groups of poultts received reference cod liver oil. The levels fed of each supplement were adjusted somewhat from those used in the first experiment in order to facilitate quantitative comparisons. In addition, higher levels of reference cod liver oil were fed because of the evidence that 200 units did not produce maximum bone ash in the first experiment. Details and results of experiment 2 are presented in table 4.

TABLE 4
Summary of experiment 2.

GROUP	SUPPLEMENT PER 100 GM. BASAL DIET			MOR- TALITY DUE TO RICKETS %	RESULTS AT 4 WEEKS OF AGE			
	Source	mg.	A.O.A.C. units		No. of poultts	Body weight gm	Tibia weight gm.	Tibia ash %
1	None	0	0	88	1	137	.7106	28.3
2	Reference	348	40	10	9	226	.6112	33.5
3	cod liver	522	60	0	8	261	.7121	38.7
4	oil	696	80	0	8	295	.8676	40.5
5		870	100	0	10	284	.8411	42.6
6		1217	140	0	9	279	.8208	46.4
7		1739	200	0	10	302	.9825	47.1
8		2609	300	0	8	302	.9278	47.3
9	Irradiated	93	39	0	9	231	.6465	40.8
10	animal	140	58	0	8	264	.7394	46.2
11	sterol	186	77	0	9	237	.6829	49.1
12		233	97	0	5	287	.8478	49.1
13	Irradiated	93	35	0	7	203	.5869	39.1
14	7-dehydro-	140	52	0	8	234	.6563	42.5
15	cholesterol	186	70	0	7	282	.8747	47.6
16		233	87	0	7	252	.7505	49.1
17	Fortified	133	57	0	8	230	.6210	41.2
18	sardine	222	96	0	9	228	.6564	44.7
19	oil	311	134	0	8	283	.8650	47.5
20		444	191	0	9	326	.9388	49.8

The data representing the bone ash response obtained in experiments 1 and 2 are presented graphically in figures 1 and 2, respectively, the percentage bone ash being plotted against the number of A.O.A.C. units fed of each supplement.

From a study of the results of these experiments it was quite clear that the vitamins D in the two irradiated products were much more

effective in promoting calcification in turkey poults than those of the fish oils when compared on the basis of the A.O.A.C. chick units fed. The results of the two experiments agreed very well.

An inspection of the graphs indicates that the response curves of the two irradiated products have a different slope from the response curves of the fish oils. It is obvious, therefore, that in attempting to give numerical expression to the differences in efficacy under these conditions a different value will be obtained for every level of response at

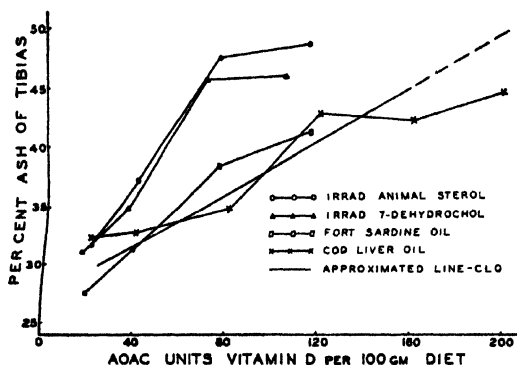


Fig. 1 Bone ash responses of poults fed vitamin D from different sources. Experiment 1.

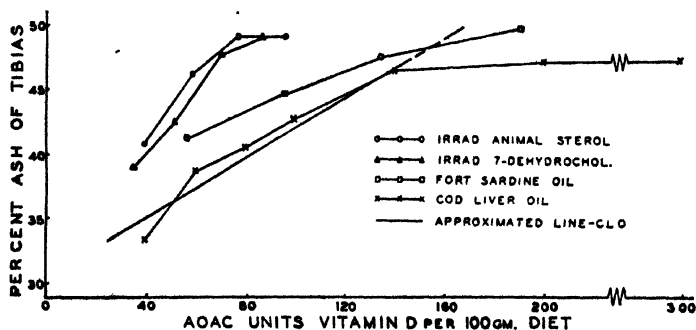


Fig. 2 Bone ash responses of poults fed vitamin D from different sources. Experiment 2.

which the comparison is made. For the present purpose a straight line of best fit has been drawn (by inspection) through the cod liver oil responses in each experiment. By placing on these approximated lines the equivalent bone ash responses of the other supplements the corresponding units have been read for each level fed. By comparing these "response" units with the number of A.O.A.C. units actually fed the

average efficacy ratio of each supplement as compared with the reference oil was calculated. These calculations for both experiments have been summarized and averaged in table 5.

TABLE 5

Efficacy ratio of the three supplements compared with reference cod liver oil.

SUPPLEMENT	GROUP NO.	TIBIA ASH %	A.O.A.C. UNITS FED	A.O.A.C. UNITS FROM C.L.O. LINE	EFFICACY RATIO CLO = 1
Experiment 1					
Irradiated animal sterol	8	31.5	19	38	2.0
	9	37.4	39	91	2.3
	10	47.7	77	183	2.4 ¹
	11	48.7	116	192	1.7 ¹
					Average 2.1
Irradiated 7-dydro-cholesterol	12	31.0	17	33	1.9
	13	34.8	35	68	1.9
	14	45.8	70	160	2.3 ¹
	15	46.1	105	170	1.6 ¹
					Average 1.9
Fortified sardine oil	16	27.4	19
	17	31.3	38	35	0.9
	18	38.3	76	99	1.3
	19	41.2	115	125	1.1
					Average 1.1
Experiment 2					
Irradiated animal sterol	9	40.8	39	87	2.2
	10	46.2	58	134	2.3
	11	49.1	77	158	2.1
	12	49.1	97	158	1.6 ¹
					Average 2.1
Irradiated 7-dehydro-cholesterol	13	39.1	35	73	2.1
	14	42.5	52	106	2.0
	15	47.6	70	145	2.1 ¹
	16	49.1	87	158	1.8 ¹
					Average 2.0
Fortified sardine oil	17	41.2	57	91	1.6
	18	44.7	96	120	1.3
	19	47.5	134	145	1.1 ¹
	20	49.8	191	164	0.9 ¹
					Average 1.2

¹ Value obtained by extending approximated straight line for the cod liver oil.

By this procedure it will be seen that the efficacy ratio for the irradiated animal sterol averaged 2.1 in both experiments, that of the irradiated 7-dehydrocholesterol 1.9 and 2.0, and that of the fortified sardine

oil 1.1 and 1.2. Obviously, for the reasons already mentioned, the ratios calculated at the high bone ash responses are relatively less valuable from a quantitative standpoint since it was only by extrapolating the straight line of the cod liver oil beyond the actual values given by this supplement that comparisons could be made at these levels. Actually, the reference oil, even when fed at 300 units per 100 gm. of diet never gave as high bone ash values as the other supplements. Consequently, if one compares the efficacy of the supplements at the level required to give maximum calcification, values appreciably greater than the averages given in table 5 would be indicated.

This suggests that turkeys, at least under the conditions of these experiments, utilize certain forms of vitamin D with degrees of efficacy that differ from chickens and exhibit a higher degree of species specificity than chickens. Certainly, it can be said that the A.O.A.C. chick unitage is not necessarily a true measure of the value of a vitamin D source for turkeys.

The results here reported confirm those of Jukes and Sanford ('39) in regard to the relative efficacy of reference cod liver oil and fortified sardine oil. It is to be remembered, however, that Jukes and Sanford ('39) used U.S.P. reference cod liver oil no. 1. While reference cod liver oil is measurably less effective than fortified sardine oil, on the basis of chick unit content, the slopes of their response curves are similar whereas both are distinctly different from the slopes of the irradiated products curves. If reference cod liver oil and fortified sardine oil can be assumed to be representative of fish oils generally, then 200 units per 100 gm. of diet from such sources may be considered an approximation of the "requirement" of turkey poults. In contrast, approximately 80 units from irradiated animal sterol or irradiated 7-dehydrocholesterol would satisfy the "requirement".

This interpretation may explain the results of Carver and Rhian ('42). Possibly the reinforced cod liver oil used by them contained an irradiation product since concentrates of irradiated animal provitamins are used in preparing such blends. It is, of course, also a possibility that fish oils from different species or from the same species at different times may vary greatly in the efficacy for turkeys of their vitamins D.

It is more difficult to explain Hammond's ('41) results with reference cod liver oil. Since his paper was submitted for publication in August, 1940, it may be assumed that he used reference cod liver oil no. 1, the same that was used by Jukes and Sanford ('39), yet he reported good protection with a level of 80 units per 100 gm. of diet. It is well known that the calcium and phosphorus contents of the diet in-

fluence the calcification of the bones but this would not seem to resolve the difficulty since Hammond's diet, while containing more calcium and phosphorus than the diet used in these experiments, contained less of both than the diet used by Jukes and Sanford. The question arises if, by chance, the "vitamin D-free commercial concentrate" of vitamin A used by Hammond may actually have carried some vitamin D. Unfortunately, there was no group of poults in his experiment that received the basal diet without the addition of a vitamin D concentrate.

SUMMARY

1. Different levels of vitamin D from four different sources, namely, U.S.P. reference cod liver oil no. 2, a sardine oil fortified with fish liver oils, an irradiated animal sterol, and irradiated 7-dehydrocholesterol were fed to poults as supplements to a rickets-producing diet during the first 4 weeks of life. All supplements were fed on the basis of their A.O.A.C. chick unit potency.

2. There were distinct differences in efficacy among the supplements, suggesting that the poult utilizes certain forms of vitamin D with degrees of efficacy that differ from the chick and in this response exhibits a higher degree of species specificity than the chick.

3. The vitamin D of the two irradiated products was distinctly more efficacious, on the basis of chick units fed, in increasing the ash content of the bones than the vitamin D of reference cod liver oil. Comparing the results obtained over the entire range of bone ash responses, this difference in efficacy is of the order of 2 to 1. While the fortified sardine oil was measurably more efficacious than the reference cod liver oil, it gave a response curve more nearly like that of cod liver oil than like the irradiated products.

4. It is concluded that the vitamin D potency expressed in A.O.A.C. chick units is not necessarily a true measure of the value of a vitamin D supplement for turkeys.

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THE PYRIDOXINE CONTENT OF FRESH, PASTEURIZED, EVAPORATED, AND DRIED MILK¹

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There are only a limited number of reports in the literature concerned with the pyridoxine content of foods. Perhaps this is caused by the fact that the place of pyridoxine in human nutrition is still not clearly defined or by the fact that the available methods of assay have been time-consuming and often inaccurate. Teply, Strong and Elvehjem ('42) have investigated the pyridoxine content of wheat and recently both Atkin and associates ('43) and Stokes and coworkers ('43) have presented several values for cereals and cereal products. Henderson, Waisman and Elvehjem ('41) have reported values for the pyridoxine content of meat. Some of the papers mentioned have also included one or more values for the pyridoxine content of fresh milk. However, the only values on processed milk are given in the paper by Atkin et al. ('43) and since the value given for evaporated milk is nearly 50% lower on a reconstituted basis than the values for fresh milk given in the same paper, a processing loss is suggested. Since a loss of this magnitude is not consistent with our knowledge of the stability of pyridoxine toward heat and oxidation, an investigation of the pyridoxine content of fresh and processed milk has been undertaken.

The microbiological method of Stokes and associates ('43) using the "pyridoxinless" mutant, no. 299 of *Neurospora Sitophila* was chosen for the investigation. The organism does not respond to pseudopyridoxine and gives reasonable agreement with rat assays on many materials. Furthermore, the method does not require any special apparatus and is sufficiently simple so that with a large autoclave and an incubating room ten to sixteen samples can be assayed simultaneously.

¹ The author wishes to thank the officials of the Pet Milk Company for releasing this report for publication and especially Dr. E. A. Louder, Technical Director, for his part in releasing the report as well as his supervision of the research program. He also wishes to acknowledge the assistance of Mr. H. E. O. Heineman in reviewing the manuscript, and the kindness of Dr. J. T. Stokes, Merck & Co., for supplying the culture of the organism used.

In the selection of samples for assay it was not possible to obtain entirely comparable samples of the various kinds of milk. The fresh samples were pooled samples from several farms. The pasteurized milk was from two plants, one small and one fairly large plant. The evaporated milk samples were from several plants scattered through California, Idaho, Illinois, Kansas, Kentucky, Missouri, Ohio, Virginia, and Wisconsin. Because of the wide geographic distribution of the plants and because each can of evaporated milk is an extremely accurate sample of the pooled processed milk from many farms, the values for evaporated milk are probably fairly representative of the pyridoxine content of milk throughout the United States as determined by the neurospora method. All evaporated samples were irradiated and contained 135 I. U. of vitamin D per reconstituted quart. The dry skim

TABLE 1
Pyridoxine content of fresh and processed milk samples

PRODUCT	NUMBER OF SAMPLES ASSAYED	MG. PYRIDOXINE ¹ PER LITER FRESH OR RECONSTITUTED MILK		AVE. MG. PYRIDOXINE ¹ ON UNDILUTED BASIS PER LITER
		Average	Range	
Fresh milk	10	0.67	0.48-0.95	0.67
Pasteurized milk	10	0.65	0.47-0.90	0.65
Irradiated evaporated milk	22	0.73	0.51-1.05	1.46
Dry skim milk	10	0.66	0.31-1.14	PER KILOGRAM 6.8
Dry whole milk	6	0.65	0.40-1.12	5.0
Average (unweighted)		0.67		

¹ Pyridoxine is reported as such. To convert to pyridoxine hydrochloride divide by 0.82.

milks were from four plants in Michigan, Utah, and Wisconsin. The dry whole milks were from a single plant. All the samples were collected in the fall, and most of them during October and November when fall pastures were still available throughout much but not all the areas from which samples were drawn. Assays were started on the processed milks within a few weeks after preparation.

The results of the investigation are recorded in table 1. The agreement between the average values on the various types of milk is very good. This indicates that there are no significant losses of pyridoxine in the preparation of the pasteurized, irradiated evaporated or dry milk. Because the samples are not entirely comparable and because the method of assay is not highly precise, it is impossible to say whether small losses may or may not occur.

Most of the reports found in the literature record only a few values for milk and these fall within the range of values in table 1. The values obtained by Atkin et al. ('43) with their yeast method, while within this range, are somewhat lower, especially since their values are in terms of pyridoxine hydrochloride rather than pyridoxine. Whether the difference between the values is merely normal variation or difference in the methods of assay is not clear. The one value obtained by rat assay and reported by Henderson, Waisman and Elvehjem ('41) is higher but when converted to a pyridoxine basis the value, 1.1 μg per milliliter is within the upper limits of the range reported in table 1. Stokes and coworkers ('43) assayed two samples of milk by the neurospora method and found values of 0.58 μg . per milliliter for each. This value is in favorable agreement with the average reported in table 1.

The reason for the wide range of the values in table 1 is not clearly understood but can probably be largely assigned to normal variation. However, we must also consider that the method of assay used is not highly precise. Stokes, Larsen, Woodward, and Foster ('43) suggest an experimental error of 15% for the neurospora method. When the method is applied to milk in our laboratory the results are somewhat more variable. When pyridoxine was added to twelve samples of fresh, evaporated, and dry whole milk and carried through the entire procedure an average total recovery of 86% was obtained. The values ranged between 80 and 93% with the single exception of one 72% recovery. The reason for these low recoveries is not apparent. A single recovery on a non-milk product (corn germ) was excellent.

Milk has often been classed as a food of low pyridoxine content. This is true when it is compared on a fresh or fluid basis with fresh meats or with air dry cereals. When compared on a dry matter basis, the pyridoxine content of milk falls in the same range as the values for cereals given by Stokes et al. ('43) and by Teply and coworkers ('42). On the same basis milk is about $\frac{1}{2}$ to $\frac{1}{3}$ as rich in pyridoxine as most meat according to the data given by Henderson, Waisman and Elvehjem ('41).

SUMMARY

1. The pyridoxine content of cow's milk has been investigated and an average value of 0.67 mg. per liter has been found for fifty-eight samples.
2. There were no significant losses of pyridoxine in the preparation of the pasteurized evaporated or dried milks assayed.
3. On a dry basis milk compares favorably with cereals in pyridoxine content and contains one-half to one-fourth as much as most meats.

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THE RETENTION OF VITAMINS IN PORK HAMS DURING CURING ¹

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The retention of thiamine, riboflavin and nicotinic acid during storage, curing and cooking of pork hams was reported in a recent paper by Schweigert et al. ('43 a). In this work the percentage retention of the vitamins during curing was calculated on the basis of the residual solids content (obtained by subtracting the moisture and fat percentage from 100) of the meat before and after treatment. Shortly after publication, Rice ² suggested that the figures given for the vitamin retention during curing might be low due to differences in salt content of the fresh and cured hams. The protein content of the ham samples used in the previous experiments was determined and the per cent of protein in the fresh and cured hams was 14.5% and 16.0%, respectively. The residual solids content of the fresh and cured hams was 15.1% and 18.9%, respectively, Schweigert et al. ('43 a). The difference between the per cent of residual solids and protein, therefore, was 0.6% in the fresh ham and 2.9% in the cured ham. If this difference was due entirely to salt, then an error was present in our results. In order to check this difference the per cent retention was calculated on the basis of the vitamin content per gram of protein in the fresh and cured hams.

To repeat this experiment four additional pairs of hams were studied. These hams were analyzed for their vitamin content, and the vitamin retention was calculated on the basis of original weight of the fresh ham and the final weight of the cured ham. For comparison the vitamin retention was also calculated on the basis of the vitamin content per gram of protein in the fresh and cured hams.

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²Personal communication from E. E. Rice, Swift and Company. We are deeply indebted to Dr. Rice for calling this possible error to our attention.

EXPERIMENTAL

Four hog carcasses of approximately the same size were selected for the study. The right and left hams from each carcass were removed and trimmed similarly. They were weighed immediately and the right and left hams were alternated for curing and for fresh samples. The hams used in the curing experiment were cured commercially. The hams were first injected with pickling solution equivalent to 8% of their original weight and placed in a pickling solution for 13 days. They were removed from the pickle and placed in a smoke house until

TABLE 1

Vitamin retention during curing of pork hams calculated on basis of fresh and cured weights.

HAM SAMPLE	HOG NO. 1		HOG NO. 2		HOG NO. 3		HOG. NO. 4	
	fresh	cured	fresh	cured	fresh	cured	fresh	cured
Weight (gm.)	5655	5535	6065	5945	5260	5045	5880	5765
Thiamine in meat ($\mu\text{g./gm.}$)	7.68	6.04	7.05	5.62	6.06	5.12	9.00	7.50
Thiamine in meat (total mg.)	43.5	33.4	42.8	33.4	31.8	25.8	52.8	43.2
Per cent thiamine retained	77		78		81		82	
Riboflavin in meat ($\mu\text{g./gm.}$)	2.13	1.99	1.98	2.07	2.43	2.43	2.02	2.05
Riboflavin in meat (total mg.)	12.0	11.0	12.0	12.3	12.8	12.3	11.9	11.8
Per cent riboflavin retained	92		102		96		99	
Nicotinic acid in meat ($\mu\text{g./gm.}$)	27.7	25.3	15.0	15.3	26.3	22.3	16.7	22.0
Nicotinic acid in meat (total mg.)	157	143	91	91	138	113	99	127
Per cent nicotinic acid retained	91		100		82		128	

the internal temperature of the ham reached 148° F. After curing the final weight of the cured ham was about 96% of the original weight of the fresh ham.

The fresh and cured hams were prepared for analysis as described by Schweigert et al. ('43 a). The protein nitrogen was determined by the Kjeldahl method, and the per cent of protein was calculated by multiplying the per cent of nitrogen by 6.25. The thiamine content of the samples was determined by the method of Hennessy ('42) with

TABLE 2

Vitamin retention during curing of pork hams calculated on the basis of protein content.

HAM SAMPLE	HOG NO. 1		HOG NO 2		HOG NO 3		HOG. NO. 4	
	fresh	cured	fresh	cured	fresh	cured	fresh	cured
Per cent protein	16.25	15.60	13.0	12.6	16.0	15.1	14.8	14.6
Thiamine (μ g./gm. meat)	7.68	6.04	7.05	5.62	6.06	5.12	9.00	7.50
Thiamine (μ g./gm. protein)	47.2	38.8	54.2	44.8	37.9	33.9	60.8	51.4
Per cent thiamine retained	82		83		89		85	
Riboflavin (μ g./gm. meat)	2.13	1.99	1.98	2.07	2.43	2.43	2.02	2.05
Riboflavin (μ g./gm. protein)	13.1	12.8	15.2	16.5	15.2	16.1	13.6	14.0
Per cent riboflavin retained	98		108		106		102	
Nicotinic acid (μ g./gm. meat)	27.7	25.3	15.0	15.3	26.3	22.3	16.7	22.0
Nicotinic acid (μ g./gm. protein)	170	163	115	122	164	148	113	149
Per cent nicotinic acid retained	96		106		90		132	

modifications by McIntire et al. ('43), the nicotinic acid by the method of Snell and Wright ('41), with modifications by Krehl et al. ('43), and riboflavin by the fluorometric method of Conner and Straub ('41) with modifications described by Andrews ('43).

All of the vitamin analyses were made directly on the samples without drying. The per cent of vitamin retention was calculated in two ways: (1) by dividing the total milligrams of the vitamin in the cured meat by the total milligrams of the vitamin in the fresh meat (table 1); (2) by dividing the vitamin content per gram of protein in the cured meat by the vitamin content per gram of protein in the fresh meat (table 2).

DISCUSSION

When the vitamin retention in the cured hams which were used in the previous experimental work was calculated on the basis of vitamin content per gram of protein, the per cent retentions were 84% for thiamine, 96% for nicotinic acid and 104% for riboflavin. These figures for vitamin retentions are higher than the retentions, 73% for thiamine, 84% for nicotinic acid and 92% for riboflavin, which we reported when the vitamin retention was calculated on the basis of residual solids. The original and final weights were not available for these hams, consequently a comparison of retentions could not be made on the basis of weight change during curing.

The riboflavin contents of the fresh hams, 1.98–2.43 $\mu\text{g.}$ per gram of fresh ham, are of the same magnitude as previously reported by McIntire et al. ('43), Schweigert et al. ('43a), and Cheldelin and Williams ('42). The thiamine values ranged from 6.06 to 9.0 $\mu\text{g.}$ per gram of fresh ham. This range is low compared to the range of thiamine values for fresh hams and loins, 7.4 to 15.2 reported by McIntire et al. ('43), and 9.8 $\mu\text{g.}$ per gram reported by Cheldelin and Williams ('42). The nicotinic acid values ranged from 15.0 to 27.7 $\mu\text{g.}$ per gram of fresh ham which are also lower than most hams previously analyzed. These values emphasize the rather wide variations in vitamin content per gram of fresh tissues which have been observed by Rice et al. ('43), McIntire et al. ('43a) and Schweigert et al. ('43a). The nutritional history of the animal may play an important role in causing variations in vitamin contents of animal tissues (Schultz et al., '39; Schweigert et al., '43 b; and Miller et al., '43), as well as variations in fat content, age of the animal and other factors.

The per cent of vitamin retentions during curing of pork hams when calculated on the basis of net weight (table 1) averaged 80% for thiamine, 97% for riboflavin and 100% for nicotinic acid. The nicotinic

acid retention of 128% observed in hog no. 4 is obviously due to an error of some kind. A complete repetition of the analysis showed that the error was not due to the nicotinic acid determination. The most probable explanation is variation in the nicotinic acid content in this particular pair of hams although the thiamine and riboflavin retentions in this pair of hams closely parallel the retentions which were obtained in other hams. The per cent of retention when calculated on the basis of the vitamin content per gram of protein averaged 85% for thiamine, 104% for riboflavin and 106% for nicotinic acid. The cured hams are all slightly lower in protein than the fresh hams which may be due to small losses of soluble nitrogenous materials during the curing of the hams.

The best method of calculating the per cent of vitamin retention seems to be on the basis of the original and final weights of the samples tested. It is the most direct method since it does not involve additional determinations and calculations which is the case when the protein content is used as the basis for calculation.

SUMMARY

1. The thiamine, riboflavin and nicotinic acid retentions during curing of pork hams have been studied using two methods of calculation.
2. When calculated on the basis of the total vitamin content of the fresh and cured hams, 80% of the thiamine, 97% of the riboflavin and 100% of the nicotinic acid were retained.
3. When calculated on the basis of the vitamin content per gram of protein in the fresh and cured hams, 85% of the thiamine, 104% of the riboflavin and 106% of the nicotinic acid were retained.

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STUDIES OF PANTOTHENIC ACID DEFICIENCY IN DOGS

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THREE FIGURES

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In the course of studies of pantothenic acid deficiency in dogs, it was found that pups survived depletion approximately 1 month, whereas adults could survive 6 months or longer¹ (Seeler and Silber, unpublished). Schaefer, McKibbin, and Elvehjem ('42) reported that growing pups have a greater requirement for pantothenic acid than adult dogs, and Unna and Richards ('42) observed a decrease in requirement with increase in age in rats. In the above dog studies it was impossible to ascertain the degree of pantothenic acid deficiency prior to the stage of collapse. Further studies therefore seemed indicated, with attempts to correlate excretion and tissue levels of pantothenic acid with other signs which might permit a more accurate determination of the onset of the deficiency.

EXPERIMENTAL

A total of twenty-two dogs were used. These included three litters of pups and one litter of young dogs. The basal diet consisted of vitamin-free casein (30%), dextrose (41%), hydrogenated vegetable fat (21%), corn oil (0.15%), cod liver oil (2%), Osborne-Mendel salt mixture (3%), and bone ash (2.85%). All dogs received 50 mg. alpha tocopherol every 2 weeks orally. All dogs also received the following supplement daily added to the first portion of the diet consumed in the morning: thiamine, riboflavin and pyridoxine, 1 mg. each; niacin, 20 mg.; choline chloride and inositol, 200 mg. each. In addition to the above supplement one group of control dogs received 40 mg. calcium pantothenate and 5 gm. dried beef liver² daily, regardless of body weight. Another group received the calcium pantothenate but no dried beef liver.

¹ One adult dog has been receiving the deficient diet since July, 1940, and is still maintaining its weight (November, 1943).

² The liver contained approximately 200 μ g. pantothenic acid, 80 μ g. riboflavin, 6.5 μ g. thiamine, 25 μ g. biotin, and 30 μ g. folic acid per gram.

Prior to depletion all dogs were given vermifuge and fed the basal diet plus all the supplements including calcium pantothenate and dried beef liver for 2 weeks; that is, the pups were given the supplemented basal diet from the age of 6 weeks to 8 weeks, and the adults received the same ration from the age of 6 to 6½ months in order to accustom them to the diet and to insure saturation with pantothenic acid at the beginning of the experiment.

At the end of this 2-week equilibration period, the calcium pantothenate and the liver were omitted from the supplement fed to five pups and two adults, and the liver alone was omitted from the supplement given to seven other pups and two adults. The six remaining pups continued to receive both calcium pantothenate and liver throughout the experiment (table 1). Of these fifteen control dogs, only five were

TABLE 1
Dietary regimen and weights of depleted dogs and controls.
(CaPa = calcium pantothenate)

DOG NO.	LITTER NUMBER	BASAL DIET	ADDITIONS TO THE SUPPLEMENT	INITIAL WEIGHT IN KILO.	TERMINAL WEIGHT IN KILO.	AGE IN MONTHS
206 M	1	Ad libitum	None	2.5	6.4 ¹	5
210 F	1	Ad libitum	None	3.1	5.2	4
212 M	1	Ad libitum	None	3.1	4.1	4
213 M	2	Ad libitum	None	4.6	6.6 ¹	5
215 F	2	Ad libitum	None	3.4	8.2 ¹	5
201 M	3	Ad libitum	CaPa	2.4	10.0	5 ²
202 F	3	Ad libitum	CaPa	2.7	8.8	5 ²
203 M	3	Ad libitum	CaPa	3.3	10.1	5 ²
		Paired with				
207 F	1	206	CaPa	2.7	6.8	5
208 M	1	210	CaPa	2.7	5.2	4
216 F	2	213	CaPa	4.2	7.5	5
217 M	2	215	CaPa	3.7	9.3	5
211 F	1	206	CaPa and liver	1.9	6.1	5
209 M	1	210	CaPa and liver	3.5	6.3	4
218 M	2	213	CaPa and liver	4.1	8.8	5
214 F	2	215	CaPa and liver	3.2	8.9	5
204 M	3	202	CaPa and liver	1.7	8.1	5 ²
207 F	3	203	CaPa and liver	3.7	10.3	5 ²
63 M	4	Ad libitum	None	17.2	14.5	10
73 M	4	Ad libitum	None	14.8	15.4	9½
		Paired with				
65 M	4	63	CaPa	16.2	13.9	10
71 M	4	73	CaPa	13.7	14.8	9½

¹ Pups 206, 213 and 215 received CaPa orally after 56-70 days on depletion.

² Pups 201 to 205, inclusive, survived beyond 5 months. Four of them are still living after 2 years on the same diet.

allowed to eat ad libitum; the other ten were fed only as much as their paired litter mates on the "depletion diet" voluntarily consumed.

For the pantothenic acid determinations, 24-hour urine specimens were collected under toluene in amber bottles, blood samples were taken from the jugular vein after overnight fast, and fresh tissues were taken at autopsy. The microbiological assay of Pennington, Snell, and Williams ('40) as modified by Silber and Mushett ('42) was employed for the pantothenic acid determinations.

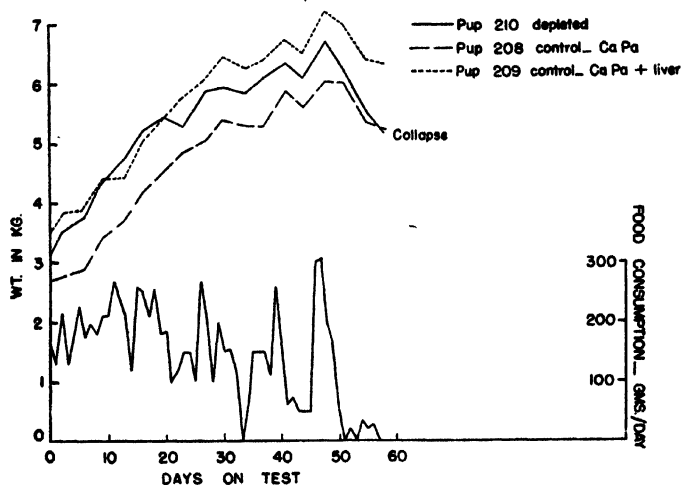


Figure 1

RESULTS

Within 2 to 3 weeks after withdrawal of calcium pantothenate, the pups showed an erratic food intake, whereas it required 7 to 8 weeks to produce a similar effect in the depleted adults. A decrease in food consumption was noted as the depletion progressed (figs. 1, 2). Two of the pups collapsed after 2 months and were sacrificed. At this time the other three in this group were given daily oral doses of 4 mg. calcium pantothenate per kilogram and showed simultaneous increases in food intake and growth (fig. 2). A similar response to oral therapy had been observed in two adult dogs in a previous study. Within a 10-week period these animals made weight gains of 57% and 113%, respectively.

After 7 to 8 weeks on the depletion diet, three of the pups became irritable and weak. One of the young adults evidenced a similar condition after 3 months. The irritability was particularly evident when the dogs were grasped at the flank. Spasticity was observed in the hind

legs during the last week on the depletion diet. Although the pups and adults on paired feeding, due to the restricted food intake, lost weight to about the same extent as the depleted dogs, they remained active and had voracious appetites. The ad libitum control pups maintained adequate food consumption and growth (fig. 3) and appeared to be normal.

Weekly examinations of the dogs revealed no evidence of gross changes in the mouth, eyes, ears, anus, or skin. The hair of the depleted dogs appeared somewhat coarser than that of the controls, with

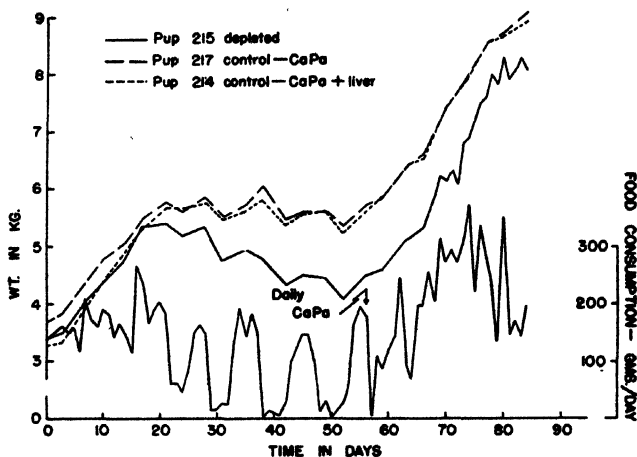


Figure 2

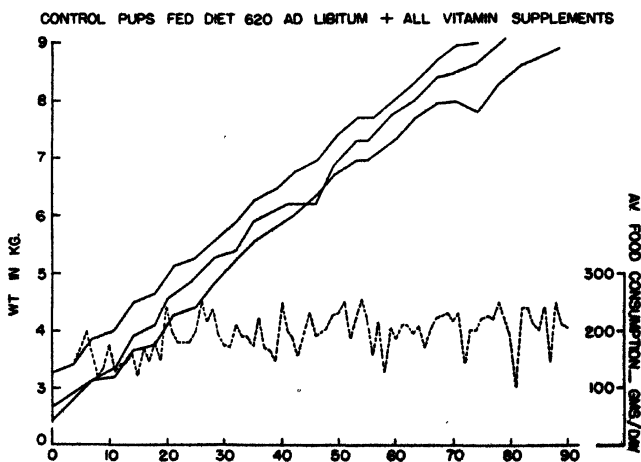


Figure 3

those receiving liver supplement possessing the smoothest coat, but there was no indication of alopecia, graying, or other skin disorder. Diarrhea was rarely observed in pups receiving liver and only occasionally in all other pups except two on depletion diet which had a loose or watery stool almost every day.

At the time of collapse, the corneal reflex was sluggish, the mouth was open with excessive salivation, and when aroused the pups were extremely weak and irritable. They behaved as though they could not see well, stumbling into and snapping at stationary objects.

After 52-54 days on test, the depleted pups and their controls showed no indication of liver damage when the bromsulphalein dye retention test was used. The older dogs also showed no evidence of dye retention when tested after 11-12 weeks on experiment.³ In spite of these negative results, some of the pups were found upon autopsy to have fatty livers.

Samples of gastric juice were taken from fasting pups $\frac{1}{2}$ hour after subcutaneous administration of 0.1 mg. histamine per kilogram (after 55 days on test). Four hundred to 1170 m. equiv. free acid and 450 to 1210 m. equiv. total acid per 100 ml. gastric contents were found, with no difference between depleted dogs and their controls.³

The total lipid and cholesterol contents of the blood of the deficient pups and adults were investigated by Scudi and Hamlin ('42) and found to be lower than in the controls and increased after oral administration of calcium pantothenate.

Gross examination of the organs of the dogs revealed no differences between depleted dogs and their controls with the exception of the livers. The livers of depleted dogs and, to a lesser degree, those of the controls not receiving a liver supplement in their diets had a yellowish appearance, due to accumulation of fat. This was confirmed by microscopic examination of the tissue. Fatty livers were also described by Schaefer et al. ('42). The fatty livers were not caused by inanition because the paired feeding controls which received the 5 gm. dried liver supplement did not show this condition. Moreover, the finding of less severe fatty livers among the controls which were not fed liver indicates that an other factor (or factors) may be involved.

Excretion of pantothenic acid in urine and feces

The pantothenic acid excretion in the urine decreased strikingly the first day after withdrawal of calcium pantothenate (table 2). There-

³ We are indebted to Dr. D. Tennent and Mr. S. Kuna for their assistance in making these tests.

after, excretion in the pups remained above normal for 3 weeks and then decreased until the animals collapsed. In the adults, which did not reach the state of collapse even after 13 weeks of depletion, the rate of pantothenic acid excretion remained relatively constant throughout this period and was in the range of nine dogs maintained on stock diet (0.04 mg. per day). That the feeding of large amounts of calcium pantothenate before giving the dogs the depletion diet was responsible for this sustained urinary excretion is indicated by the fact that two other dogs, not previously dosed, consistently excreted only 0.002 to 0.010 mg. per day after receiving the same deficient diet for 2 to 3 months. Furthermore, pups which were not given massive doses of

TABLE 2
Urinary excretion of pantothenic acid during depletion.
(Data expressed in milligrams per 24 hours
after intervals indicated)

PUP NO.	BEFORE DEPLETION	1 DAY	1 WEEK	2 WEEKS	3 WEEKS	5 WEEKS	7 WEEKS	9 WEEKS
206	11.5	0.24	0.04	0.09	0.07	0.010	0.052	0.008
210	10.4	0.83	0.08	0.12	0.23	0.017
212	12.2	0.18	0.06	0.10	0.08	0.007	0.025
213	5.7	0.29	0.10	0.10	0.09	0.013
215	10.4	0.20	0.13	0.12	0.27	0.050	0.003	0.007
Average	10.0	0.35	0.10	0.13	0.15	0.021	0.024	0.008

ADULT NO	BEFORE DEPLETION	1 DAY	2 WEEKS	3½ WEEKS	5½ WEEKS	7½ WEEKS	9½ WEEKS	13 WEEKS
63	0.5	0.20	0.050	0.040	0.014	0.040	0.050	0.055
73	3.3	0.12	0.080	0.035	0.026	0.022	0.070	0.033
Average	1.9	0.16	0.065	0.038	0.020	0.031	0.060	0.045

calcium pantothenate prior to receiving the deficient diet, have been found to collapse after only 1 month. This also indicates that the calcium pantothenate dosage had a prolonged effect, which not only maintained excretion levels but even permitted appreciable growth during the first 3 weeks of depletion (figs. 1, 2).

The paired-feeding control pups, which continued to receive 40 mg. calcium pantothenate daily, excreted an average of 14.0 mg. urinary pantothenic acid per day. The adults excreted an average of 0.7 mg. per day. Aqueous extracts of 24-hour fecal specimens from depleted, treated (40 mg. calcium pantothenate per day), and stock diet dogs assayed, respectively, 0.05–0.5 mg. per day, 10.0–20.0 mg. per day, and 1.3–2.8 mg. per day. The fecal weight from depleted dogs, however,

was only 5–30 gm. per day, whereas stock diet dogs excreted 200–600 gm. in the same period, so there is little difference between these two groups when the comparison is made on the basis of fecal pantothenic acid concentration. The fecal pantothenic acid assay appears to be valid because baryta treatment quantitatively destroyed the pantothenic acid content of such extracts.

TABLE 3

*Pantothenic acid content of tissues of dosed, depleted, and normal dogs.
Data expressed in micrograms per gram tissue (wet weight)*

TISSUE	PUPS DEPLETED 210, 212	CONTROL PUPS 207, 208, 209 211	CURED PUPS 206, 213, 215	DEPLETED YOUNG ADULTS 63, 73	CONTROL YOUNG ADULTS 65, 71	THREE STOCK DOGS ¹
Liver	25	73 (2)	65	35	55	62
Kidney	42	68	47	49	63	47
Brain	16	43	30	24	41	22
Heart muscle	18	31	35 (2)	23	50	20
Pancreas	24	30	18 (2)	28	22	20
Leg muscle	5	23	22	7	26	11
Adrenal	14 (1)	28	23 (2)	24	38	23 (2)
Spleen	7	10	9	9	7	8
Lung	5	7	8	12	8	6
Blood	0.1 (4)	0.4 (13)	0.3 (2)	0.1	0.3	0.2 (12)

Numbers in parentheses indicate number of cases included in the average value given, wherever it differs from the number of animals listed.

Tissue levels of pantothenic acid

Nine or ten tissues from each of sixteen dogs were assayed for pantothenic acid as shown in table 3. With few exceptions, the tissue levels of the depleted animals were lower than those of the controls. The fact that the young adults were not as severely depleted as the pups was borne out by the less pronounced differences in tissue levels. When the tissue levels of the experimental dogs are compared with those of dogs on stock diet, two points are evident: (1) the control dogs which had received 40 mg. calcium pantothenate daily had more pantothenic acid in their blood, kidney, brain, and muscle tissues than the stock diet dogs which had an intake of 1.5–3.0 mg. dietary pantothenic acid per day, and (2) the depleted dogs had lower than normal levels only in liver, muscle, brain and blood.

DISCUSSION

Although significant decreases have been found in the pantothenic acid levels of blood, liver, muscle, brain, urine, and feces of severely

depleted dogs, the possibility of revealing a deficiency state by any single determination seems slight. The differences between the levels of stock diet dogs and depleted dogs are not great enough to exclude biological variation and experimental error as complicating factors.

The pathological findings of Schaefer et al. ('42) that pantothenic acid depleted pups developed a mottled thymus and hemorrhagic degeneration of the kidney have not been observed in our experiments. It should be noted that our diets and vitamin supplements differed in several respects from theirs.

The fact that adult dogs can survive depletion much longer than pups might be the result of (1) a lower requirement for maintenance than for growth, (2) ability to utilize significant amounts of pantothenic acid already in the tissues, and (3) intestinal synthesis.

When calcium pantothenate was administered before a depletion period was started, the blood and urinary pantothenic acid levels as well as growth remained essentially normal during the first 3 weeks. When, on the other hand, depletion was started without previous calcium pantothenate dosage, pups collapsed after 1 month and adults excreted only 0.002–0.010 mg. per day after only 2 to 3 months. A prolonged effect of massive dosage is indicated.

SUMMARY

1. The first manifestations of pantothenic acid deficiency in pups were an erratic, decreasing appetite and a decrease in urinary excretion of the vitamin. Growth and food intake showed parallel decreases prior to collapse after 2 months. The deficiency responded to oral therapy.

2. Older dogs showed a delayed effect on appetite; the urinary pantothenic acid content was decreased, but was still essentially normal after 3 months depletion. This is in agreement with the fact that the pantothenic acid requirement of older dogs is lower.

3. The concentration of pantothenic acid in blood and other tissues of pantothenic acid depleted dogs was below that of controls dosed with large amounts of calcium pantothenate; however, when compared with stock diet dogs, the only significantly low pantothenic acid levels were found in liver, muscle, brain and blood. Repeated oral dosage with calcium pantothenate increased tissue levels above those found in dogs on stock diet and appeared to have a delaying effect on the progress of subsequent depletion.

4. The only gross pathological changes observed as a result of the deficiency were fatty livers. These were observed primarily in the

depleted pups but also, to a lesser degree, in the control dogs on the same diet supplemented with calcium pantothenate. This was not true of control dogs which received a dried beef liver supplement in addition. Spasticity was observed in the hind quarters during the last week of the deficiency.

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ERRATUM

Berryman, George H., and Paul E. Howe

A short method of calculating the nutritive value of diets, concluded

J. Nutrition, vol. 27, no. 3, March, 1944

Correct as follows:

TABLE 4.

1. "Tomatoes" instead of "Totatoes".
2. Value for fat per lb. of citrus fruits should be "1" gm. instead of "47".
3. Value for Vitamin A per lb. of citrus fruits changed from 96 to 380 per lb. (IU.)

TABLE 5.

1. Value for nicotinic acid per lb. of vegs. leafy green and yellow changed from .27 mg. to 2.7.
2. Value for Vitamin A per lb. of citrus fruits changed from 90 to 360 (IU.)

A STUDY OF THE RIBOFLAVIN AND THIAMINE REQUIREMENTS OF CHILDREN OF PRESCHOOL AGE

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TWO FIGURES

(Received for publication December 20, 1943)

In this study, the riboflavin and thiamine excretions of two normal 5-year-old boys were followed as the intakes of these vitamins were increased from very low to much higher levels. In addition, 1-hour fasting excretions, 4-hour and 24-hour returns of test doses of both vitamins, and the thiamine content of the blood were determined during the last period on each level of intake. It was hoped that this type of study would furnish information concerning the needs of children of this age for these vitamins as well as a comparison of the different methods for determining nutritional status with regard to riboflavin and thiamine.

METHODS OF STUDY

The subjects were two normal 5-year-old boys whose weights were 16.6 and 17.7 kg. They had been living in an institution for 3 months previous to this study and were known to have received good diets. During the period of study, they lived in the hospital but were given an unusual amount of freedom. When weather permitted, they were taken outside for play both morning and afternoon; otherwise, they played in a large, airy playroom. This schedule was considered comparable to that of the average city child of this age.

The experiment ran continuously for thirty-six 3-day periods with the exception of a 6-day period on one child. This interruption was due to an acute upper respiratory infection, coupled with a gastro-intestinal disorder; the study was resumed when he was apparently normal, as judged by a physician. Later in the study, both children had a similar disorder which also lasted for 6 days. During this time, urine collec-

¹ Quaker Oats Fellow of the American Academy of Pediatrics.

tions were made as usual, although it was necessary to change their diets. The riboflavin and thiamine intakes, however, were maintained by supplements of the pure vitamins.

Diets were given which were adequate except for their riboflavin and thiamine contents. The meal pattern used throughout the study was: Breakfast—apple juice or orange juice, oatmeal or unenriched farina,² toast, butter and jelly; Dinner—chicken, lamb or beef, rice or potato, vegetable, fruit, bread, crackers and butter; Supper—chicken, lamb, beef, peanut butter or egg, vegetable, fruit, bread and butter, jello, ice or grape juice, cookie. Only canned vegetables and fruits were used. Those included were carrots, beets, peas, tomatoes, green beans, pears, peaches, apple sauce and pineapple. The bread was made by one of us from unenriched flour, low vitamin yeast and without milk. At each level of intake, foods which furnished the desired amounts of riboflavin and thiamine, were selected from this list and arranged in three daily menus which were used in rotation as long as the subjects were on that particular level. Milk in amounts ranging from 140 to 630 gm. per day was given in all periods except those of lowest riboflavin and thiamine intakes. In addition, the diet was supplemented with 500 mg. calcium as dicalcium phosphate; 25 mg. of ascorbic acid; 6 mg. of iron as ferric pyrophosphate and 2 teaspoonfuls of cod liver oil daily until such time as adequacy in these nutrients was assured by dietary means. Servings of foods furnishing appreciable amounts of vitamins were kept constant but the consumption of such foods as bread, butter, jelly, crackers and unenriched farina was not limited.

At the end of the study the regular hospital diet, estimated to contain approximately 800 μ g. of thiamine and 1500 μ g. of riboflavin, was given ad libitum for 5 days with daily supplements of 6 mg. of riboflavin and 4 mg. of thiamine.³ Following this, the supplements were discontinued for a 2-day period before the final test doses were given.

All meals were prepared, weighed and served by one of us. At the same time samples of each food were taken for analysis. Composites representing one-fifth of the food eaten during each 3-day period, with the exception of cereals, butter and sugar, were made at the end of the period, homogenized and adjusted to approximately pH 1 by the addition of 8 ml. of concentrated hydrochloric acid per liter of suspension. Cereals were treated separately in order to rule out any untoward effect on the riboflavin assays.

² Kindly supplied by the Quaker Oats Company.

³ The riboflavin and thiamine were generously supplied by Merck and Company.

Continuous urine collections were made. Each sample was immediately preserved with toluene and acetic acid and then refrigerated. One hour specimens of urine voided before breakfast were collected on 2 or more days in the periods previous to changes in the diet and analyzed separately. Riboflavin and thiamine test doses of 75 μ g. per kilogram of body weight were given orally before each increase in vitamin intake. This amount was selected because it seemed to be one which could be used for children of all ages and weights without making the test doses excessively high in the case of large children and too low in the case of small ones. The thiamine and riboflavin test doses were given on successive days with vitamin low breakfasts. Consecutive 4-hour and 20-hour collections were made and analyzed separately. The 4-hour test dose return was considered to be the total excretion during the 4 hours immediately following the administration of the test dose; the 24-hour return was calculated by subtracting the average daily excretion of the previous period from the total excretion during the 24 hours following the test dose administration.

The riboflavin content of food composites and of urine was determined by the microbiological method (Snell and Strong, '39; Strong and Carpenter, '42; Silber and Unna, '42). A slightly different basal medium in which⁴ the amounts of peptone and glucose were double those used in standard basal medium, was used for cereal assays. Isbell, Wooley and Fraser ('41) reported that urea in amounts greater than 20 mg. per tube inhibited the growth of *lacto-bacillus casei* and caused a downward drift in the riboflavin values as the amount of urine per tube was increased. We encountered no such drifts in our assays when the urine was increased from 1 to 5 ml. per tube and interpreted this as evidence that no inhibition was taking place. Urea determinations were not made but on the basis of the protein intake, the amount of urea in the tube containing 5 ml. of urine was calculated to be 40 mg. or more.

Thiamine determinations were made by the microfermentation method⁵ (Atkin, Schultz and Frey, '39) as described by Knott, Kleiger and Torres-Bracamonte ('43):

⁴ Suggested by Dr. Harris Isbell of the U. S. Public Health Service.

⁵ After this paper was submitted for publication, an article by H. F. Deutsch ('44) appeared in which he stated that the yeast microfermentation method may give only approximate values for thiamine since other factors may also have a stimulatory effect, particularly during the pyrimidine determination. According to this, our thiamine values should be low. However, when compared with thiochrome assays on the same samples, the values obtained by the microfermentation method were usually slightly higher.

RESULTS AND COMMENT

Riboflavin

One-hour fasting excretions. After 12 to 18 days on average intakes of 250 and 275 $\mu\text{g.}$ the 1-hour fasting excretions of both subjects contained only very small amounts (1–2 $\mu\text{g.}$) of riboflavin (table 1, fig. 1). Such small excretions, both relatively and absolutely, have been interpreted as an indication of near depletion (Najjar, '42). On higher intakes the excretions increased and paralleled the intakes with the exception of the periods following the upper respiratory infections. Such a gradual rise made this test of little value in studying riboflavin needs, since as yet, the amount indicative of a good nutritional state is not known. However, its eventual use as a test of nutritional status with respect to riboflavin after standards have been established seems promising, since it showed good correlation (0.83)⁶ with the dietary intake.

Test dose returns. A comparison of the 4- and 24-hour returns of test doses of 75 $\mu\text{g.}$ per kilogram (table 2) shows that both rose gradually as the riboflavin intake was increased and that both reflected the depleting effect of the upper respiratory infections. The correlation between the 4- and 24-hour returns was 0.89 and both showed good correlations with the dietary intake (0.76 and 0.79, respectively) and with the fasting 1-hour excretions (0.92 and 0.85, respectively). Thus it seems that the 1-hour fasting excretion, the 4-hour and the 24-hour return of a test dose all reflect equally well the nutritional status with respect to riboflavin. However, since neither the 1-hour fasting excretion nor the 4-hour return of the test dose requires the determination of the excretion during a control day on the same diet, these tests would be preferable in most cases.

Daily excretions. The average daily excretions of both subjects decreased progressively at the 250- and 275 $\mu\text{g.}$ levels of intake and increased progressively at the 550- $\mu\text{g.}$ level (table 1, fig. 1); this was interpreted as evidence of a gradual depletion of tissue riboflavin at the lower and a gradual rebuilding of normal stores at the higher level. If the 550- $\mu\text{g.}$ intake had been continued for a longer period it is possible that eventually it might have supported even higher excretions.

⁶ Correlation coefficients were calculated according to the Product-Moment formula:

$$r_{xy} = \frac{\frac{\sum xy}{n} - \bar{x}\bar{y}}{\sqrt{\frac{(\sum x^2 - \bar{x}^2)}{n} \frac{(\sum y^2 - \bar{y}^2)}{n}}}$$

TABLE 1
Riboflavin and thiamine excretions and thiamine blood levels on different intakes

SUBJECT 1

PERIODS	AV. DAILY INTAKE RIBOFLAVIN			RIBOFLAVIN EXCRETION			PE- RIODS	AVERAGE DAILY INTAKE OF THIAMINE		THIAMINE EXCRETION			BLOOD THIA- MINE PER 100 ML.
	Cal- ories	Calcu- lated ¹	Deter- mined	Av. per 24 hr. in each period	4-hr. after test dose	Av. per 1 hr. fast- ing ²		Calcu- lated ¹	Deter- mined	Av. per 24 hr. in each period	4-hr. after test dose	Av. per 1 hr. fast- ing ²	
1-6	1290	300	250	50, 40, 30, 20, 20, 20	140	1	1-6	225	150	90, 20, 10, 20, 10, 30	30	3	4.9
7-12	1390	700	550	30, 40, 40, 40, 70, 70	160	4	7-16	475	475	50, 50, 40, 90, 90, 60, 40, 60, 50, 50	20	2	6.9
13-18	1310	900	700	130, 140, 130, 140, 110, 50	240	9	17-20	575	600	130, 70, 120, 110	150	3	6.3
19-26 ³	1450	1150	850	100, 70, 470, 200 180, 180, 120, 90	100	3	21-26 ³	750	700	150, 90, 190, 170, 150, 230
27-31	1370	1375	1050	90, 230, 230, 200, 210	110	13	27-31	775	700	170, 130, 100, 180, 210	150	6	8.4
32-36	1540	1850	1450	320, 340, 330, 400, 170	420	23	32-36	900	950	280, 290, 280, 370, 280	160	11	7.5
37	7500	5080	37	4800	...	2370
38	1500	990	440	35	38	800	...	950	300	17	8.4

SUBJECT 2

1-4	1520	325	275	50, 20, 20, 30	80	2	1-4	250	200	120, 30, 30, 50	70	2	3.6
5-10	1570	725	550	30, 30, 50, 60, 70, 80	150	5	5-14	500	525	40, 100, 100, 150, 90, 90, 90, 80, 60, 60	80	3	4.8
11-16	1380	900	725	110, 90, 100, 90, 100, 140	220	9	15-20	575	600	50, 130, 140, 130, 160, 120	130	4	5.3
17-26 ³	1410	1125	875	170, 160, 210, 150, 340, 440, 300, 170, 100, 110	140	7	21-26 ³	750	700	180, 180, 220, 180, 230, 120
27-31	1690	1425	1075	160, 140, 220, 250, 270	280	14	27-31	825	750	160, 180, 140, 150, 130	160	7	7.1
32-36	1520	1775	1425	470, 430 320, 490, 440	360	23	32-36	875	925	290, 310, 220, 280, 260	180	7	7.7
37	7500	5050	37	4800	...	1560
38	1500	830	440	25	38	800	...	800	300	11	6.5

¹ Both the riboflavin and the thiamine content of the diet were calculated from a table of values which was compiled after a survey of published data from many sources. Recipes were calculated and losses in cooking and canning were taken into consideration. These calculations were made for comparison with the determined values on the composites. The discrepancy between calculated and determined riboflavin values is due chiefly to the riboflavin content of the milk which was calculated as 200 µg. per 100 gm. Analyses showed it to contain approximately 180 µg. per 100 gm.

² The 1-hour fasting values represent the average of two or more determinations, carried out on successive days.

³ During periods 21 and 22 both subjects had acute upper respiratory infections.

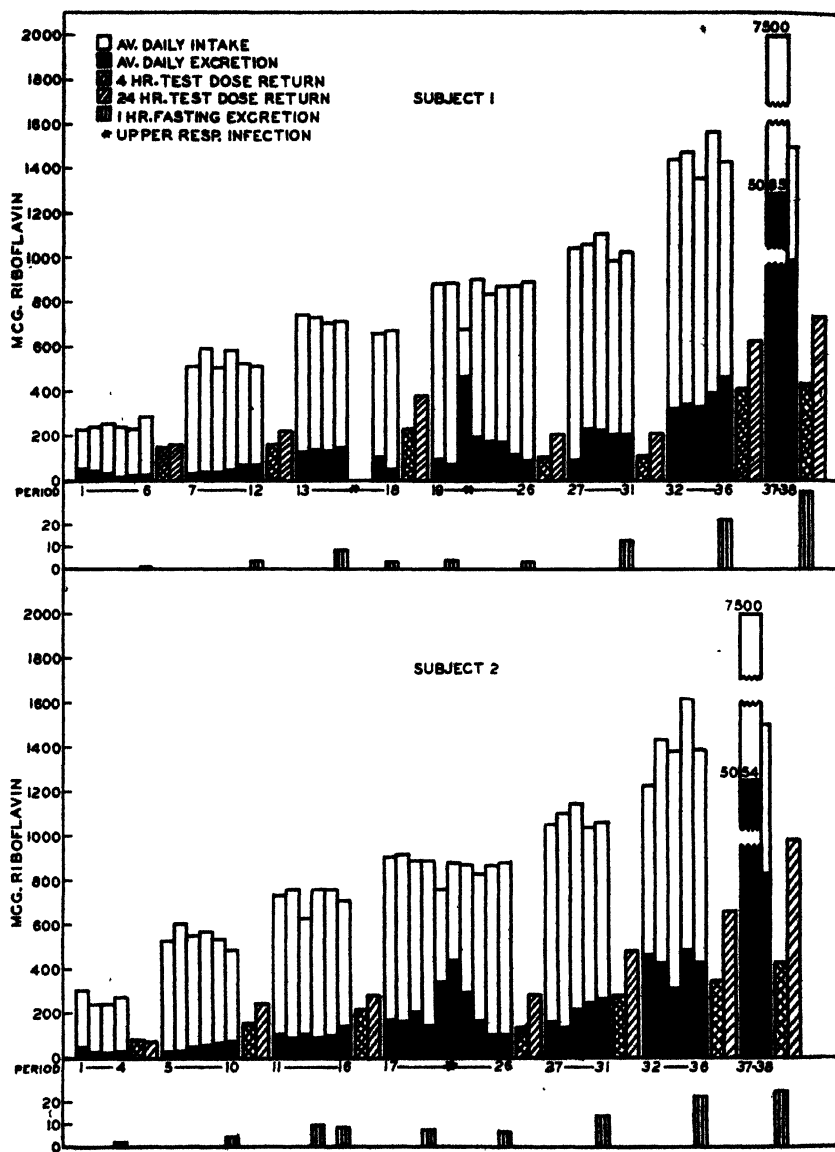


Fig. 1 Average daily excretions, test dose returns and fasting excretions of riboflavin at different intake levels.

The comparatively constant excretions, however, on the 700- and 725- μ g. intakes (0.53 mg. per 1,000 Cal.), made it seem quite certain that this amount met the requirements of these subjects, and would therefore be an adequate allowance for children of this age. When the intake was at this level, the average daily excretions were 117 and 105 μ g.; the 1-hour fasting excretions were 9 μ g.; the 4-hour returns of test doses were 20 and 17% and the 24-hour returns of test doses were 32 and 23%.

TABLE 2

Comparison of 4- and 24-hour returns of 75 μ g. per kilogram test doses¹ of riboflavin and thiamine

SUBJECT	RIBOFLAVIN			THIAMINE		
	Average dietary intake	Test dose return		Average dietary intake	Test dose return	
		4 hr. ²	24 hr. ³		4 hr. ²	24 hr. ³
	μ g.	%	%	μ g.	%	%
1	250	11	13	150	2	13
2	275	6	6	200	5	9
1	550	13	18	475	1	5
2	550	12	19	525	6	12
1	700	20	32	600	13	22
2	725	17	23	600	10	32
1	850	9	17	700
2	875	11	22	700
1	1050	8	17	700	12	17
2	1075	20	36	750	12	18
1	1450	33	50	950	13	22
2	1425	26	47	925	13	29
1	1500 ⁴	35	59	800 ⁴	23	57
2	1500 ⁴	32	71	800 ⁴	22	55

¹ Test doses for subject 1 were approximately 1250 μ g.; for subject 2, 1350 μ g.

² Total excretion during 4 hours following test dose administration with a vitamin low breakfast.

³ Total excretion during 24 hours following test dose administration less the average daily excretion of the previous period.

⁴ Calculated intakes following the 5 days when supplementary vitamins were given.

The extremely high excretions of riboflavin of subject 1 during period 21 and of subject 2 during periods 21, 22 and 23 were obviously due to the acute upper respiratory infections with accompanying elevations of body temperature which probably caused a coincident lack of protein deposition. This would be in agreement with the findings of Sarett et al. ('42, '43) that the riboflavin excretion of dogs and rats varied inversely with the protein intake and that protein deposition was necessary for riboflavin retention in the liver. During subsequent periods in

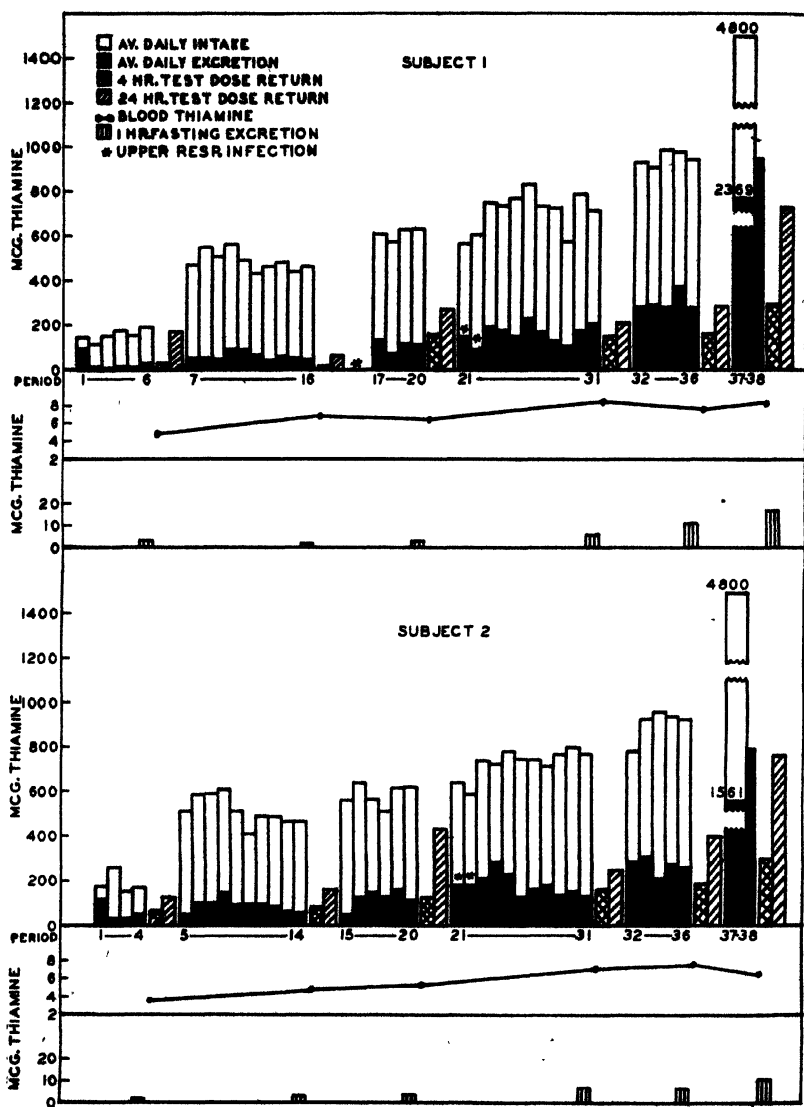


Fig. 2 Average daily excretions of thiamine, test dose returns, fasting excretions and blood thiamine at different intake levels.

which both children were apparently normal in every respect and when compensatory protein deposition might have been expected the riboflavin excretions were low. This effect was also noted after a similar illness of subject 1 which occurred following period 16. Collections were discontinued during the time of the illness but his excretion of riboflavin was lower in periods 17-20 than it had previously been on a similar intake. These lowered excretions continued for 12 to 15 days after the children had apparently recovered which suggests the need for increased riboflavin intake after this type of illness.

Average daily excretions of over 300 μ g. on 1450- and 1425- μ g. intakes (periods 32 to 36) and of over 5000 μ g. on a 7500- μ g. intake in period 37 made us believe that the tissues of these subjects were saturated with riboflavin on the 1450- and 1425- μ g. intakes. This viewpoint was further substantiated by the similarity of the test dose returns on the two levels of intake.

Riboflavin requirement. A riboflavin intake of approximately 0.50 mg. per 1,000 Cal., and amount one-third less than the 0.75 mg. per 1,000 Cal. provided by the dietary allowances of the Food and Nutrition Board of the National Research Council, seemed to be ample for the subjects of this study. At this level of intake (0.53 mg. per 1,000 Cal.) the fasting 1-hour excretions were 9 μ g., and 4-hour test dose returns approximately 20%. Excretions of this magnitude, therefore, might be considered indicative of satisfactory nutritional status with respect to riboflavin until more data are available.

Thiamine

One-hour fasting excretions. The 1-hour fasting thiamine excretions followed the same general pattern as those of riboflavin (table 1, fig. 1). They were from 2 to 4 μ g. on intakes of less than 700 μ g. but increased to 6 and 7 μ g. at that level. Like the riboflavin excretions, they correlated well (correlation coefficient 0.85) with the dietary intake; this made it seem that this test reflected the nutritional status with respect to thiamine.

Test dose returns. The returns of thiamine test doses of 75 μ g. per kilogram (table 2) were small on the low thiamine intakes but both the 4- and 24-hour returns showed marked increases at the 600- μ g. level. No further increases of any magnitude occurred until after the subjects had received supplementary thiamine in period 37. No evidence of the depleting effects of the upper respiratory infections was shown, as was the case with respect to the riboflavin test dose returns. This, how-

ever, was not surprising, since the daily excretions of thiamine during periods 21 and 22 were not abnormally high.

The correlation between the 4-hour and the 24-hour returns was 0.91 and both showed good correlations (0.86 and 0.82, respectively) with the fasting 1-hour excretions as well as with the intakes (0.87 and 0.92, respectively). Again, as in the case of riboflavin the decision as to which of these three tests to use would depend on the particular situation rather than the superiority of any one of them.

Blood thiamine. The thiamine blood levels were slightly lower than 5.0 $\mu\text{g.}$ per 100 ml. of whole blood on the 150- and 200- $\mu\text{g.}$ intake levels. This figure was suggested as optimal for infants by Knott ('42). However, since Benson et al. ('42) found an average blood level of 7.8 $\mu\text{g.}$ per 100 ml. in a group of forty-five normal children and since we found that the average blood thiamine of a group of thirty-nine normal children was increased from 5.5 to 7.3 $\mu\text{g.}$ per 100 ml. when the diet was improved,[†] it is possible that for good nutrition a level of approximately 7.0 $\mu\text{g.}$ per 100 ml. is desirable. The two subjects in this study had values for blood thiamine of this magnitude on intakes of 700 and 750 $\mu\text{g.}$, respectively.

The correlation coefficients of the blood thiamine with the fasting 1-hour excretions, 4-hour and 24-hour returns of test doses and average intakes were 0.66, 0.58, 0.40 and 0.45, respectively. Therefore, it seems as though less confidence can be placed in this test than in the excretion tests as a measure of nutritional status.

Daily excretions. The average daily excretions of thiamine adjusted to a changed intake quite rapidly (fig. 1) after which they remained fairly constant at all levels of intake. The per cent of ingested thiamine excreted increased gradually with the intake and when the latter reached 600 $\mu\text{g.}$, 18% was excreted by subject 1 and 20% by subject 2. On intakes of 700 and 750 $\mu\text{g.}$, the per cent excreted was only slightly higher (23%) but on the next level studied (950 and 925 $\mu\text{g.}$), it increased to 30%. When the intakes were increased to 4800 $\mu\text{g.}$, 49% was excreted by one subject and 33% by the other.

Thiamine requirements. If an excretion of 20% or more of the daily thiamine intake can be considered indicative of adequacy, as suggested by Benson et al. ('42), it would seem that 600 $\mu\text{g.}$, satisfied the needs of these subjects fairly well. The sudden increase in test dose returns at this level, gives it further support. On the other hand, at the 700- to 750- $\mu\text{g.}$, intake level, the per cent of dietary thiamine excreted daily was not much above that at the 600- $\mu\text{g.}$; the 1-hour fasting excretions showed

[†] Unpublished data.

their first significant increase at this level of intake and the thiamine blood levels were higher. This indicated that 750 $\mu\text{g.}$, of thiamine (approximately 0.50 mg. per 1,000 Cal.) would probably be a safer allowance for children of this age until further data are available. This amount was that recommended by the Food and Nutrition Board of the National Research Council for children of this age. It also agrees fairly well with the 0.45 mg. per 1,000 Cal. suggested by Benson et al. ('42a) for children and by Williams et al. ('43) as the minimum requirement of the adult. At this level, the daily excretions were 23% of the intake; the fasting 1-hour excretions were 6 and 7 $\mu\text{g.}$; the blood thiamine levels were 8.4 and 7.1 $\mu\text{g. \%}$; the 4-hour test dose returns were 12% and the 24-hour test dose returns 17 and 18% of the test dose.

SUMMARY AND CONCLUSIONS

The average daily urinary excretions of riboflavin and thiamine of two preschool children were determined on various levels of intake. One-hour fasting excretions, 4- and 24-hour returns of test doses of both vitamins and thiamine content of the blood were determined at each intake level.

The 1-hour fasting excretions and the 4- and 24-hour test dose returns were found to correlate well with each other and with the intake. There was less evidence of correlation of blood thiamine with both the intake and the other tests of nutritional status. It seems, therefore, that the 1-hour fasting excretion and the return of an oral test dose can be used equally well in determining the nutritional status with respect to riboflavin and thiamine but that the blood thiamine level is less reliable.

The riboflavin requirement was considered to be the lowest level at which the daily excretions neither decreased nor increased progressively. In the case of thiamine, the requirement was based on an average daily excretion of 20% of the intake, decided increases in the fasting 1-hour excretions and test dose returns, and blood levels of 7.0 $\mu\text{g.}$ or more per 100 ml. Riboflavin and thiamine intakes of approximately 0.50 mg. per 1,000 Cal. satisfied these criteria and therefore appeared to meet the needs of the subjects used in this study.

Until such time as more data are available, the return, in 4 hours, of 20% of a test dose of riboflavin and of 12% of a test dose of thiamine or fasting 1-hour excretions of 9 $\mu\text{g.}$ of riboflavin and 6 $\mu\text{g.}$ of thiamine might be considered indicative of satisfactory nutritional status with respect to these vitamins.

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VITAMIN INTERRELATIONSHIPS

III. INFLUENCE OF SUB-OPTIMUM DOSES OF THIAMINE ON URINARY EXCRETIONS OF RIBOFLAVIN ¹

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Recently Sure and Ford ('42) reported that in thiamine deficiency there is a pronounced disturbance in riboflavin metabolism. Such observations were made on animals that were thoroughly depleted of thiamine, hence losses of weight were encountered in some of the avitaminotic animals, although in some groups the normal animals restricted in food to that consumed by the pathological litter mates actually lost more weight than the vitamin B₁ deficient rats. These weight changes, not given in the paper referred to, were taken in consideration in arriving at the conclusion drawn that the marked disturbance in riboflavin metabolism produced by thiamine deficiency is due mainly to poor retention and not necessarily to body tissue catabolism.

Since border-line rather than marked thiamine deficiencies are more commonly found in this country, it was thought of interest to investigate the influence of chronic thiamine deficiency on riboflavin utilization. Since urinary excretions gave an index of retention and fecal riboflavin excretions did not contribute any material information to the picture in our previous study (Sure and Ford, '42), this investigation was limited to only urinary excretions. Previously we found large individual differences in fecal excretions of riboflavin and it was, therefore, felt an additional set of data would not appreciably influence the relative results on retention, if noteworthy changes were observed in urinary excretions of riboflavin on different doses of thiamine.

The experiments were carried out in four series, the results of which are summarized in table 1.

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TABLE 1
Influence of sub-optimum thiamine intake on urinary excretions of riboflavin

SERIES	RATION	MENTA- FOLEUM PERIOD	EXPERIMENTAL PERIOD	NO. OF AN- IMALS	DAILY VITAMIN INTAKE	TOTAL FLAVIN INTAKE	CHANGES IN BODY WEIGHT	ENDO- GENOUS EXCRETION IN URINE ^a	PER CENT OF ENDO- GENOUS FLAVIN EXCRETED IN URINE
		days	days		μg.	μg.	gm.	μg.	
A	5357	7	58th to 65th	4	1 to 2	140	- 13.8	43.8	31.3
A	5357	7	58th to 65th	4	3	140	- 4.5	29.4	21.0
A	5357	7	58th to 65th	4	4	140	- 4.2	33.3	23.1
A	5357	7	58th to 65th	4	5	140	- 3.5	29.0	20.7
A	5357	7	58th to 65th	4	6	140	- 3.3	19.7	14.1
A	5357	7	58th to 65th	4	11	140	- 0.3	25.4	18.1
A	5357	7	71st to 78th	4	2 to 4 ^a	70	- 2.0	33.7	48.1
A	5357	7	71st to 78th	4	3	70	- 1.3	18.4	26.3
A	5357	7	71st to 78th	4	4	70	- 2.0	16.2	23.1
A	5357	7	71st to 78th	4	5	70	- 1.0	14.9	21.3
A	5357	7	71st to 78th	4	6	70	- 1.3	11.9	17.0
A	5357	7	71st to 78th	4	11	70	+ 1.0	13.9	19.8
A	5388	13	80th to 93rd	4	4	130	+ 6.5	34.5	24.7
A	5388	13	80th to 93rd	4	8	130	+ 7.5	19.6	15.2
A	5388	13	80th to 93rd	4	12	130	+ 8.3	11.8	9.1
A	5388	13	80th to 93rd	4	16	130	+ 7.3	8.4	6.5
A	5388	13	80th to 93rd	4	20	130	+ 7.8	8.8	6.7
A	5388	13	80th to 93rd	4	50	130	+ 8.5	16.9	13.0
B	5388	13	180th to 193rd	4	1 to 3	260	- 7.3	95.9	36.9
B	5388	13	180th to 193rd	4	4	260	+ 2.7	52.3	20.1
B	5388	13	180th to 193rd	4	6	260	+ 3.0	23.3	8.9
B	5388	13	180th to 193rd	4	8	260	- 0.3	25.2	9.7
B	5388	13	180th to 193rd	4	10	260	+ 1.3	20.3	7.8
B	5388	13	180th to 193rd	4	20	260	+ 2.8	22.0	8.5
B	5388	9	195th to 204th	4	1 to 3	90	- 2.3	31.8	35.3
B	5388	9	195th to 204th	4	4	90	- 0.3	23.9	26.5
B	5388	9	195th to 204th	4	6	90	- 0.5	6.9	7.6
B	5388	9	195th to 204th	4	8	90	+ 1.0	6.8	7.3
B	5388	9	195th to 204th	4	10	90	+ 0.0	6.5	7.2
B	5388	9	195th to 204th	4	20	90	- 1.8	3.5	3.9

^a Corrected for the amount excreted on a riboflavin-deficient ration. The figures in this table represent averages per animal from data secured on four animals.

^b Animals were in depleted thiamine state and it was necessary to administer a minimum of 2 to 4 μg. of this vitamin daily to prevent polyneuritis and insure adequate food consumption to circumvent rapid catabolism.

MODIFIED PROCEDURE FOR DETERMINATION OF RIBOFLAVIN
IN RAT'S URINE

Before presenting the results of this investigation a brief description should be given of the modified analytical procedure used for the determination of riboflavin in the urine. The urine is collected in amber-colored bottles containing 10 to 12 ml. N/5 H_2SO_4 to which has been added 15 drops of toluene. The urine is analyzed four times weekly. During 3 days of the week the urine, adjusted to a pH of about 1 and preserved with toluol, is kept in a refrigerator. In the mornings the funnels are washed with a fine spray of enough distilled water to allow 200 ml. dilution for a 48-hour sample and a 100 ml. dilution for a 24-hour sample. Ten ml. aliquots are transferred into 50-ml. amber-colored Erlenmeyer flasks. The pH is adjusted to about 4.5 with 2N sodium acetate buffer, using bromo-cresol-green as an outside indicator. One drop of a saturated solution of KMnO_4 is added, the flasks are rotated gently several times during 2 minutes, which is sufficient time for the oxidation of foreign fluorescent substances to take place. One drop (and only occasionally 2 drops) of a 3% solution of H_2O_2 removes the excess of KMnO_4 and the diluted urine solution clears up without foaming. Five ml. of distilled water are now added, so that the cuvette for the Pfaltz and Bauer fluorophotometer contains exactly 15 ml. total volume. The instrument is set so that the galvanometer reads 25 for 1 μg . riboflavin. The whole operation is so rapid that we generally run twelve determinations consecutively. The riboflavin is then reduced to the colorless form with 10 to 20 mg. pure sodium hydrosulfite crystals. The latter reagent need not be weighed but can be roughly estimated by the amount that can be picked up on the tip of a spatula. The riboflavin in the urine is computed from a calibrated curve which is linear. All riboflavin determinations are carried out with the curtains of the laboratory drawn, so as to exclude as much light as possible.

We find the procedure described above much more accurate and rapid than the one used previously (Sure and Ford, '42). The two main advantages are that it prevents foaming of the diluted urine sample, and the decomposition of the aqueous solution of sodium hydrosulfite, used previously, during the determinations is circumvented.

SERIES A EXPERIMENTS

In this series there were six male and eighteen female rats. They were started on experiments when they were 35 days of age and weighed 47 to 65 gm. each. The investigation was carried out on twenty-four animals in four groups of six rats in each group. The animals were

given ration 5357 of the following percentage composition: casein (vitamin-free)² 18; agar-agar, 2; salts no. 1 (Sure, '41), 4; butter fat, 10; polished rice, 30; and cerelose, 36. Thiamine was administered daily in graduated doses, and according to the set up, four animals were available for each dose. The first animal received the smallest dose and the amount of feed it consumed was allotted to the rest of the five animals in each group. The thiamine daily doses were 0, 2, 3, 4, 5, and 10 μ g., respectively. After 4 weeks the first animal was given 1 μ g. B₁ daily. To these doses one must add the 1 μ g. contributed daily by the polished rice in the ration. Other vitamin supplements were given daily as follows: 20 μ g. riboflavin, 20 pyridoxine, 6 mg. choline chloride and 100 μ g. calcium pantothenate, later increased to 200 μ g. As a source of vitamins A and D, 3 drops of halibut liver oil were given once weekly to each animal. The same vitamin supplements were administered to the rest of the animals in series B following. Since the animals in series A were on isocaloric diets, they were equalized with respect to weights during the three metabolism periods, the weights ranging from 110 to 120 gm. each.

The first 7-day metabolism study was carried out on the fifty-eighth to sixty-fifth days of the experiment. From table 1 it is apparent that only the animals which received the 1 to 2 μ g. thiamine daily showed a greater excretion of riboflavin in the urine. However, since the rats which received these thiamine doses lost more weight than the rest of the animals in this group, the greater excretion of riboflavin may be attributed to losses from the tissues produced by body catabolism. This picture was revealed from consideration of the effect of the 20 μ g. daily dose of riboflavin. However, during the next 7-day metabolism period, when the daily dose of riboflavin was reduced to 10 μ g., 6 to 11 μ g. of thiamine produced the lowest excretion of riboflavin in the urine (table 1). The animals which previously received 1 to 2 μ g. daily were in an advanced avitaminotic state and had to be given 3 to 4 μ g. daily to prevent polyneuritis and marked anorexia, and because of that depleted state, were excreting more riboflavin in the urine than the litter-mate controls, which were receiving 3 μ g. thiamine daily. The weight changes during the second as well as third metabolism period were negligible, which eliminates body tissue catabolism as a factor in the interpretation of the results. Further evidence of a thiamine-riboflavin interrelationship in chronic B₁ deficiency became evident in the third metabolism period of 10 days when this group of animals was given purified synthetic diet 5388, which is the same as ration 5357,

² SMACO.

modified by replacement of 30% polished rice with an equivalent amount of cerelese. It will be noted that on this purified diet free from natural foods the optimum thiamine dose for 10 μ g. riboflavin is 12 to 16 μ g. Increasing the thiamine dose from 4 to 16 μ g. decreased the riboflavin excretion in the urine, calculated as per cent of total intake, from 24.7 to 6.5%. In other words, on the 16 μ g. daily dose of thiamine a little less than $\frac{1}{4}$ of the riboflavin intake was excreted in the urine as on the 4 μ g. daily dose of thiamine. However, the increase in urinary excretion of riboflavin when the daily thiamine was increased from 20 to 50 μ g. was not confirmed by later experiments.

SERIES B EXPERIMENTS

This series of experiments was conducted with twelve male and twelve female rats. They were started when 35 days old, weighing 42 to 68 gm. each. The animals were placed on ration 5388 and during the first 111 days feed was allowed ad libitum, and the daily thiamine doses were 0 to 2, 2, 3, 4, 5, and 15 μ g., respectively. The experiments were carried out as in series A, with four groups of animals, six in each group. From the one hundred and eleventh day on isocaloric diets were fed and the daily thiamine doses were changed to 1 to 3, 4, 6, 8, 10, and 20 μ g. In spite of differences in weight precipitated by the ad libitum feeding the first 111 days of the entire 204 days of the experimental period, the chronic avitaminosis produced by administration of inadequate amounts of thiamine, was responsible for large excretions of riboflavin in the urine on daily thiamine doses below 6 μ g. (table 1). This occurred when the daily dose of riboflavin was either 10 or 20 μ g.

In the first metabolism period, on the 20 μ g. daily dose of riboflavin the increase of the daily intake of thiamine from 1-3 to 10 μ g. reduced the urinary excretion of riboflavin, expressed as per cent of intake, from 36.9 to 7.8, or about one-fifth. Similarly, in the second metabolism experiment, when the daily riboflavin dose was 10 μ g., the increase in the daily intake of thiamine from 1-3 to 6 μ g. reduced the urinary excretion of riboflavin from 31.8 to 6.9% of the total intake. It is thus evident that in chronic thiamine deficiency, uncomplicated by body tissue catabolism, greater excretions of riboflavin occur on sub-optimum intakes of vitamin B₁. Such greater excretions of riboflavin, as shown in a recent publication (Sure and Ford, '42) represent relatively lesser retention of this vitamin; hence it follows that on sub-optimum doses of thiamine which produce a chronic B₁ avitaminosis, there is a poor retention of riboflavin.

DISCUSSION

It is apparent from the results of the experiments submitted in this communication that a definite thiamine-riboflavin interrelationship exists in chronic as well as in acute thiamine deficiency (Sure and Ford, '42). During 2 months of inadequate thiamine intake no significant changes were observed in urinary excretions of riboflavin on a 20- μ g. daily dose of this vitamin, but great excretions of riboflavin in the urine occurred on a 10- μ g. daily dose. However, on a 6-month sub-optimum thiamine intake large excretions of urinary riboflavin occurred on a 20- as well as a 10- μ g. daily dose of riboflavin. The question arises as to the possible significance of these findings in human experience. Ferrebee and Waisman ('43) attempted to answer this question but their experiences with rats were limited to only a few weeks thiamine depletion and they were dealing with acute and not with chronic thiamine avitaminosis. Also, the clinical results submitted by these investigators on four patients could hardly be considered as convincing, particularly since these patients, although suffering from malnutrition, were not representative chronic or advanced cases of thiamine deficiency. It is suggested that, if any relationship between chronic thiamine deficiency and riboflavin retention exists in the human, evidence should be obtained from numerous cases of chronic border-line deficiencies in anorexias of children, polyneuritis of pregnancy, alcoholic polyneuritis, and in patients suffering from cardiovascular dysfunction associated with B₁ avitaminosis (Weiss and Wilkins, '36 and '37; and Weiss, '40).

SUMMARY

Chronic thiamine deficiency produces great losses of riboflavin in urine, uncomplicated by body tissue catabolism, and hence lowers the retention of the latter vitamin.

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ASSOCIATIVE DYNAMIC EFFECTS OF PROTEIN, CARBOHYDRATE AND FAT¹

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TWO FIGURES

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It is the understanding of the authors that a nutrient fed alone is never metabelized alone, and that the apparent energy expense of utilization, or heat increment, or dynamic effect, of a nutrient is much affected (1) by the nutrient combination in which it is metabolized, (2) by the method of disposition of the absorbed nutrients by the experimental subject, and (3) by the technic of measurement of the dynamic effect as representing a single feeding of a test nutrient or a difference between higher established states of nutrition.

In this light the present investigation was conducted to determine the dynamic effects in mature, male albino rats of protein, carbohydrate and fat, singly and in four combinations, all fed as supplements to the same quantity of the same nutritively complete, basal, maintenance diet.

The advantages of the general procedure followed, as compared with the measurement of dynamic effects of single test meals fed to animals in a post-absorptive state, are that the results are more representative of nutritive practice; they are not confused with the dynamic effects of katabolized body nutrients; and they are both more significant and apt to be more accurate, since they are measured as the difference between the amounts of heat produced at established planes of metabolism, without need for concern as to the beginning, the maximum and the end of the dynamic effect as of the single test meal.

For the information of readers who are accustomed to determining dynamic effects of feeds from single portions of the test substances fed to animals in a post-absorptive state, and who therefore expect to

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observe a marked rise and subsequent fall in heat production while the animal utilizes the test substance, the authors advise that the difference between the heat production of fasting rats and rats receiving feed is small, especially as compared with the comparable value for ruminants, and that they have a great volume of unpublished data showing that when rats are fed twice per day there is no falling off in the heat production, as indicated by the elimination of carbon dioxide, at the end of 7-hour periods of observation under the conditions of the present study. Therefore, when dynamic effects of nutrients are determined by the comparison of the heat production of the same group of rats established consecutively on two planes of nutrition above energy equilibrium, the variations in the heat production during the two periods of observation are considered to cancel out by subtraction.

A conceivable minor imperfection in the determination of dynamic effects at planes of nutrition above maintenance, however, is that they do not include the amount of energy which would be required to eliminate the nutrients retained as part of body tissue.

The literature relating to the dynamic effects of nutrients has not been reviewed in connection with this paper because it has been recently reviewed by Wilhelmj ('35), Borsook ('36), Murlin ('39), and Kriss ('41), and because there has been no previous study of the present subject by the general procedure followed.

EXPERIMENTAL

The general technic employed, which is derived from Armsby's studies of the energy metabolism of cattle, depending on the comparison of the heat production from established states of nutrition, has been used at this institute since 1902 — recently in studies of the dynamic effects of nutrients for the albino rat (Kriss et al., '34; Kriss, '38; Forbes et al., '39b), and is here utilized, with special adaptations, in the further investigation of this latter subject.

An advantage of this procedure is that conditions which affect the rate at which the diet is metabolized are without effect on the heat increment since these conditions prevail both at the beginning and the end of the period of observation.

In recognition of the considerable individual variation in the heat production of animals, twelve experimental subjects were used for each observation. The experimental program, as shown in table 1, was so arranged that periods compared were immediately contiguous. The subjects were of exactly the same age and approximately the same body weight at the beginning of each unit of the investigation. Each

comparison of heat production was made between two sets of observations on a single group of rats, but a different group of rats was used for each such comparison. Also the subjects were trained for the respiration experiments by subjection to the experimental conditions in blank runs on at least 2 days prior to the respiration measurements.

TABLE 1
Schedule of experimentation.

RAT NUMBERS	DAILY FOOD INTAKE	AGE OF RATS	AVERAGE WEIGHT OF RATS	DATES OF RESPIRATION MEASUREMENTS
		<i>days</i>	<i>gm.</i>	
1-12	10 gm. basal diet	170	298	Oct. 12-17
1-12	10 gm. basal + 4 gm. cerelose (corn sugar)	177	308	Oct. 19-24
13-24	10 gm. basal diet	170	282	Nov. 9-14
13-24	10 gm. basal + 3.54 gm. beef protein	177	293	Nov. 16-21
25-36	10 gm. basal diet	170	269	Dec. 7-12
25-36	10 gm. basal + 1.5 gm. lard	177	277	Dec. 14-19
37-48	10 gm. basal diet	170	286	Jan. 4-9
37-48	10 gm. basal + 2 gm. cerelose + 1.848 gm. beef protein	177	300	Jan. 11-16
49-60	10 gm. basal diet	170	297	Feb. 1-6
49-60	10 gm. basal + 2 gm. cerelose + 0.737 gm. lard	177	303	Feb. 8-13
61-72	10 gm. basal diet	170	301	Mar. 1-6
61-72	10 gm. basal + 1.848 gm. beef protein + 0.737 gm. lard	177	306	Mar. 8-13
73-84	10 gm. basal diet	170	286	Mar. 29-Apr. 3
73-84	10 gm. basal + 1.333 gm. cerelose + 1.232 gm. beef protein + 0.491 gm. lard	177	297	Apr. 5-10

The subjects were sexually mature and had passed the period of most rapid growth, but under favorable conditions were still able to retain significant proportions of their nitrogen intake, as shown in table 2.

In each unit of the investigation each of the twelve subjects was used first for individual determination of the metabolizable energy and the heat production from 10 gm. of the basal diet, followed by the same determinations with reference to this basal diet plus one of the test supplements in a quantity computed to yield, in each case, approximately the same amount of metabolizable energy. The dynamic effects of the supplements, therefore, were computed by difference between the quantities of heat produced from the basal and the supplemented diets.

The consecutive time intervals occupied by these measurements were as follows: (a) Basal diet—introductory period, 10 days; excreta

collection for determination of metabolizable energy, 8 days; respiration experiment, 2 days; (b) Supplemented diet — introductory period, 5 days; respiration experiment, 2 days; excreta collection for determination of metabolizable energy, 8 days. The special purpose of this arrangement was to provide that the measurements of the heat production on the supplemented diet should follow the measurements on the basal diet with the shortest practicable intervening interval of time.

The heat production was determined by the respiratory quotient procedure. Protein metabolism was computed from the urinary nitro-

TABLE 2
Utilization of daily nitrogen.

RAT NUMBERS	FEEDING TREATMENTS	NITROGEN INTAKE	NITROGEN DIGESTED	NITROGEN OF URINE	NITROGEN RETENTION
		mg.	%	%	%
1-12	Basal diet	365	69.0	61.7	7.3
1-12	Basal + cerelose	365	71.1	50.5	20.6
13-24	Basal diet	365	68.4	54.3	14.1
13-24	Basal + beef protein	864	82.8	63.7	19.1
25-36	Basal diet	365	68.0	69.9	— 1.9
25-36	Basal + lard	354	70.2	42.8	27.4
37-48	Basal diet	365	66.8	56.9	9.9
37-48	Basal + cerelose + beef protein	632	79.7	54.9	24.8
49-60	Basal diet	365	68.3	64.7	3.6
49-60	Basal + cerelose + lard	362	68.8	50.9	17.9
61-72	Basal diet	365	70.0	60.8	9.2
61-72	Basal + beef protein + lard	622	80.3	61.2	19.1
73-84	Basal diet	365	70.4	51.6	18.8
73-84	Basal + cerelose + beef protein + lard	543	78.4	59.3	19.1

gen. Carbon dioxide production was determined by absorption, and oxygen utilization determined by the method of Haldane as the combined weights of carbon dioxide and moisture given off minus the loss in weight of the animal.

The factors employed in this computation (Loewy and Oppenheimer, '11) were the following:

Urinary N (mg.) \times .00476 = liters CO_2 from protein.

Urinary N (mg.) \times .00594 = liters O_2 used by protein.

Urinary N (mg.) \times 26.51 = calories from protein.

Caloric values per liter O_2 , at respiratory quotients as determined, are given in Lusk's modification (Lusk, '24) of the table of Zuntz and Schumburg ('01). In those cases in which the non-protein respiratory quotient exceeded unity the caloric value of the excess CO_2 was reckoned as 1.09 Cal. per liter, as determined by Bleibtreu ('01).

The rats were fed twice a day, and heat production was measured on 2 consecutive days during 7-hour periods beginning at 8:30 A.M., about 1½ hours after the morning feeding.

The weight of the carbon dioxide absorbed, and the degree of physical activity of the experimental subject as indicated by a work adder, were recorded each hour, and the values used for heat production were selected as those representing intervals of time during which the physical activity and the heat production were at their lowest levels. The weight of carbon dioxide eliminated during the entire 7-hour period was used in computing the respiratory quotient, but only the amount eliminated during the selected intervals of quiet were used in computing the heat production. It was assumed that the total respiratory quotient was unaffected by the slight activity of the animal during the 7-hour respiration period. The definite significance of the results obtained, therefore, was limited to the selected intervals of quiet, without evidence or claim that they exactly represented the entire day of 24 hours. It is also conceded that there was probably a difference between the amounts of heat produced by the subjects while asleep and while awake but motionless. The respiration experiments were conducted with the apparatus at a temperature of $28^{\circ}\text{C.} \pm .1^{\circ}\text{C.}$, the temperature inside the respiration chamber being about 29°C.

The dates of the respiration measurements, as given in table 1, cover the intervals of 6 days during which each of the twelve rats on a dietary treatment was subjected to respiration measurements on 2 consecutive days, tests being made on four rats each day.

The basal diet was a complete commercial feed manufactured for dogs; the protein supplement was dried and extracted beef muscle; the carbohydrate supplement was a corn sugar product, called cerelese; and the fat used was commercial lard.

The differences in the body weights of the rats at ages 170 and 177 days represented in part, each, gain in body substance and in alimentary fill. Since these gains in weight were small, and since the extent, in each case, to which they represented body gain and increase in alimentary fill was not in evidence, no effort was made to correct the heat production to correspond to the same body weight in the basal and the supplemented periods.

RESULTS — DISCUSSION

Referring to table 2, the nitrogen intake in the diets was essentially the same, as also was the apparent digestibility of the nitrogen, in all diets except those containing beef muscle protein. In these cases the

apparent digestibility of the nitrogen of the diets was materially increased by the added amounts of highly digestible protein. This supplementary beef protein presumably added little either to the undigested nitrogen, or to the metabolic nitrogen, of the feces.

As compared with the nutritive status of the rats on the basal diet — with any greater amount of either nitrogenous or non-nitrogenous nutriment there was an increase in the percentage of the food nitrogen digested and in the percentage retained. Also the quantity of nitrogen in the urine was increased by all increases in dietary protein, and decreased by all increases in non-protein nutrients.

Among the diets in which the supplements contained protein, the apparent digestibility of the nitrogen varied in the order of the variation in their protein contents, in part at least as results of the decrease in the proportion of the protein eliminated as metabolic products in the feces.

The partition of the energy of the diets is represented by table 3.

The digestibility of all the dietary components was so high that there could be little difference in the digestibility of the energy of the entire diets, the extreme difference in the average daily fecal energy in the fourteen experimental periods being between 7,206 cal. and 8,529 cal. In six among the seven dietary comparisons the amount of the fecal energy from the supplemented diet was slightly greater than that from the basal diet of the preceding experimental period.

The outgo of energy in the urine was slightly but definitely higher from the diets containing the non-protein supplements, and much higher from the diets in which the supplements contained protein, than from the preceding basal diets.

In computing the metabolizable energy values of the diets a correction was made, as usual, for the non-metabolizable energy of the protein gained. This correction has been made in the caloric values of the urine as given in table 3.

The average metabolizable energy values of the diets were phenomenally consistent, the coefficients of variation of the twelve individual determinations on the seven basal diets being between 1.0% and 2.6% and, of the seven supplemented diets, between 0.8% and 1.4%.

By inspection of the seven average metabolizable energy values for the same basal diet, it will be observed that the extreme range of variation was between 30,149 cal. and 30,659 cal. in 40,090 cal. of gross energy; and a statistical study of the differences in the seven average determinations of the metabolizable energy value of the basal diet

TABLE 3
Partition of daily food energy.

RAT NUMBERS	FEEDING TREATMENTS	INTAKE			FEACES		URINE ¹		METABOLIZABLE ENERGY				HEAT PRODUCTION		ENERGY BALANCE
		Basal	Supplement	Total	cal.	cal.	cal.	cal.	%	Coefficient of variation	cal.	%	Coefficient of variation		
1-12	Basal diet	40,090		40,090		7,460	2,092	30,538	1.2	23,977	5.7	6,561			
1-12	Basal + cerelese	40,090	13,712	53,802		7,552	2,332	43,918	.6	26,753	4.6	17,165			
13-24	Basal diet	40,090		40,090		7,627	2,218	30,245	2.6	24,262	9.5	5,983			
13-24	Basal + beef protein	40,090	16,913	57,003		8,529	5,412	43,062	1.2	29,731	10.4	13,331			
25-36	Basal diet	40,090		40,090		7,409	2,074	30,607	1.0	22,208	7.6	8,399			
25-36	Basal + lard	40,090	14,186	54,276		7,819	2,225	44,232	1.0	24,474	5.4	19,758			
37-48	Basal diet	40,090		40,090		7,824	2,117	30,149	2.5	24,195	7.6	5,954			
37-48	Basal + cerelese + beef protein	40,090	15,571	55,667		7,712	4,039	43,916	.8	27,899	5.8	16,017			
49-60	Basal diet	40,090		40,090		7,504	2,071	30,515	1.3	25,097	3.8	5,418			
49-60	Basal + cerelese + lard	40,090	13,697	53,787		8,040	2,233	43,514	1.4	26,733	3.4	16,781			
61-72	Basal diet	40,090		40,090		7,206	2,225	30,659	1.0	25,656	9.2	5,003			
61-72	Basal + beef protein + lard	40,090	16,275	56,365		7,705	3,924	44,736	.8	27,495	5.5	17,241			
73-84	Basal diet	40,090		40,090		7,229	2,257	30,604	1.1	23,945	6.6	6,659			
73-84	Basal + cerelese +beef protein + lard	40,090	15,425	55,515		7,755	3,377	44,383	1.0	26,740	7.5	17,613			

¹ Corrected for the non-metabolizable energy of the protein gained.

revealed that among twenty-one possible comparisons only one appeared to be significant (odds 37 to 1).

The coefficients of variability among the measurements of the heat production were much less satisfactory. For the seven basal diets they varied between 3.8% and 9.5%, and for the seven supplemented diets between 3.4% and 10.4%.

These variations presumably resulted from differences in animal individuality; in effects of environmental conditions such as heat, light, air pressure and noise; and in the status of the subjects as to whether awake or asleep. The only way to avoid embarrassing variability among individual measurements of the heat production in experiments with animals seems to be to work with single subjects on each experimental treatment — as many experimenters have done. Heat production by animals is a highly individual and labile function.

The values for energy balance in the fourteen experimental periods should be understood as resultants of 8-day measurements of food, urine and fecal energy computed to a 24-hour basis, and measurements of the heat production during relatively small parts of 2 experimental days raised to the 24-hour basis.

The derivation of the heat increments from cerelese, beef protein and lard, singly and variously combined, as determined in each case by difference between the heat production for a basal period and for the immediately following supplemented period, is indicated by the data in table 4, and graphically in figure 1.

In this figure the numerical values above the seven graphs represent approximately equally metabolizable, daily, gross energy intake of small calories in supplements fed in addition to the basal diet. The plus and minus values for protein, carbohydrate, and fat represent *calories of increased or decreased heat production from these nutrients as results of the consumption of the nutritive supplements*, the base of comparison in each case (represented by the horizontal line across the graph) being the amount of heat produced from the protein, carbohydrate or fat of the basal diet.

For the purpose of this presentation the energy of fat synthesis is considered to be derived from the katabolism of carbohydrate.

The feeding of beef protein, cerelese and lard, individually, as supplements to a complete basal diet, had the effect in each case to increase the production of heat from the kind of nutriment represented by the supplement, these dynamic effects diminishing in the order in which the nutrients are mentioned. Cerelese spared twice as much heat production from fat as from protein; beef protein spared about the same

TABLE 4
Derivation of heat production from cerelese, beef protein and lard, singly and variously combined.

RAN NUMBERS	FEEDING TREATMENTS	HEAT PRODUCTION		HEAT PRODUCTION FROM		
		cal.	Protein	Carbo- hydrate	Fat	Fat synthesis
1-12	Basal diet + cerelese	26,753	4,884	21,538	0	331
1-12	Basal diet	23,977	5,974	15,725	2,272	6
1-12	Cerelese (by difference)	2,776	— 1,090	5,813	— 2,272	325
13-24	Basal diet + beef protein	29,731	14,599	14,270	661	201
13-24	Basal diet	24,262	5,259	16,425	2,569	9
13-24	Beef protein (by difference)	5,469	9,340	— 2,155	— 1,908	192
25-36	Basal diet + lard	24,474	4,014	15,004	5,456	0
25-36	Basal diet	22,208	6,762	14,813	584	49
25-36	Lard (by difference)	2,266	— 2,748	191	4,872	— 49
37-48	Basal diet + cerelese + beef protein	27,899	9,184	18,403	46	266
37-48	Basal diet	24,195	5,510	15,163	3,509	13
37-48	Cerelese + beef protein (by difference)	3,704	3,674	3,240	— 3,463	253
49-60	Basal diet + cerelese + lard	26,733	4,884	21,435	350	64
49-60	Basal diet	23,097	6,263	15,771	3,063	0
49-60	Cerelese + lard (by difference)	1,636	— 1,379	5,664	— 2,713	64
61-72	Basal diet + beef protein + lard	27,495	10,084	17,026	348	37
61-72	Basal diet	25,656	5,886	17,515	2,250	5
61-72	Beef protein + lard (by difference)	1,839	4,198	— 489	— 1,902	32
73-84	Basal diet + cerelese + beef protein + lard	26,740	8,540	18,069	0	131
73-84	Basal diet	23,945	4,995	17,190	1,755	5
73-84	Cerelese + beef protein + lard (by difference)	2,795	3,545	879	— 1,755	126

amounts of heat production from carbohydrate and fat; lard was highly effective in sparing heat production from protein, leaving the amount of heat produced from carbohydrate essentially unchanged.

The mixed supplements of cerelose and beef protein, and of cerelose, beef protein and lard, increased the production of heat from both protein and carbohydrate; the supplement of cerelose and lard increased the heat production from carbohydrate alone, and the supplement of beef protein and lard increased the production of heat from protein alone. All four mixed supplements, whether they contained

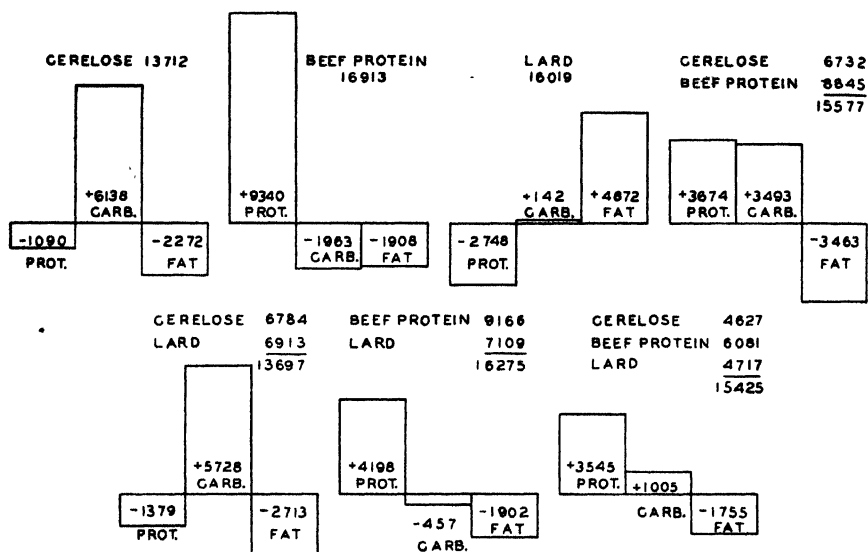


Fig. 1 The source of the dynamic effects of nutrients as affected by nutrient combination. This graph is explained in the text.

lard or not, decreased the production of heat from fat, thus emphasizing the energy reserve function of fat. The high dynamic effect of beef protein fed as a single supplement to a basal diet is not characteristic of beef protein fed as a component of a mixed supplement with cerelose or lard, or with cerelose and lard.

The energy values of the supplementary nutrients, individually and variously combined, are given in table 5. The metabolizable energy value of beef protein was found to be characteristically low, in spite of its high digestibility, because of the elimination of incompletely oxidized nitrogen compounds in the urine. Among the mixed supplements the metabolizable energy values of the three which contained

TABLE 5
Energy values of nutrients, individually and variously combined.

SUPPLEMENTARY NUTRIENTS	METABOLIZABLE ENERGY AS PER CENT OF GROSS		HEAT INCREMENTS AS PER CENT OF GROSS		HEAT INCREMENTS AS PER CENT OF METABOLIZABLE		NET ENERGY AS PER CENT OF GROSS		NET ENERGY AS PER CENT OF METABOLIZABLE	
	Computed from Experimentally determined values for components		Computed from Experimentally determined values for components		Computed from Experimentally determined values for components		Computed from Experimentally determined values for components		Computed from Experimentally determined values for components	
	%	%	%	%	%	%	%	%	%	%
Cerelose (3.42 Cal. per gram)	97.6	...	20.2	...	20.7	..	2.65	77.3	79.3	...
Beef protein (4.86 Cal. per gram)	75.8	...	32.3	...	42.7	.	2.08	43.5	57.3	...
Lard (9.46 Cal. per gram)	96.0	...	16.0	...	16.6	...	7.57	80.1	83.4	...
Cerelose + beef protein	88.4	85.9	23.8	27.2	26.9	30.8	2.02	64.6	73.1	66.4
Cerelose + lard	94.9	97.7	11.9	18.3	12.6	19.2	4.15	83.0	87.4	83.7
Beef protein + lard	86.5	82.2	11.3	24.4	13.1	28.2	4.74	75.2	86.9	66.9
Cerelose + beef protein + lard	89.3	86.7	18.1	23.1	20.3	25.9	3.59	71.2	79.7	71.2

protein were slightly higher as directly determined than as computed from the individual values for the components. The heat increments, or dynamic effects, of the nutritive supplements, as per cent of the gross energy, differed from the heat increments as per cent of the metabolizable energy mainly as influenced by the relatively low metabolizable energy value of the beef protein—the cerelese and the lard being almost completely metabolizable. The heat increment values of the mixed supplements, related either to the gross energy or the

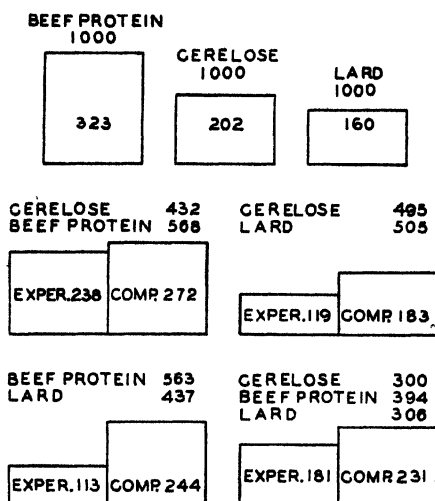


Fig. 2 The dynamic effects per thousand calories of gross energy of nutrients as affected by nutrient combination. This graph is explained in the text.

metabolizable energy, were in all cases materially lower as experimentally determined than as computed from the values for the individual components of these supplements, thus showing that nutrient combination favors efficiency in the utilization of metabolizable energy.

The heat increments, or dynamic effects, per 1000 cal. of gross energy of the individual nutritive supplements, and similar values for the four mixed supplements as directly determined and as computed from the values for their individual components, are graphically presented in figure 2.

The values above the rectangles represent in each case 1000 cal. of gross energy intake of supplementary nutrients, and the values inside the rectangles represent the dynamic effects of these nutritive supplements.

The values for dynamic effects of mixed supplements expressed as "Exper. . . ." were as experimentally determined, while those expressed as "Comp. . . ." were computed from the separately determined dynamic effects of beef protein, cerelese and lard; the amounts by which the experimentally determined values were lower than the computed values expressing the extent to which nutrient combination effected economy of utilization.

In the separate determinations of the dynamic effects of supplementary beef protein, cerelese and lard it was observed that the dynamic effect of the protein was 32%, of the carbohydrate 20%, and of the fat 16%, of their respective gross energy or fuel values; and such individual values are generally but erroneously considered to apply to normal diets in which these nutrients occur variously combined. Upon such individual values, commonly determined at planes of nutrition below energy equilibrium, depends the prevalent misconception that the dynamic effects of diets differ in accord with their protein contents.

The most efficient utilization of 1,000 cal. of food energy of the mixed supplements characterized the mixture of beef protein and lard. The dynamic effect of this combination was lower even than that of the supplement of lard alone (113 cal. compared with 160 cal. per 1000). The least efficient utilization of 1000 cal. of food energy of the mixed supplements characterized the one of cerelese and beef protein (238 cal. per 1,000). The dynamic effects of the combinations of cerelese and lard, and of cerelese, beef protein and lard, were intermediate between the extremes noted.

The well-known effect of fat to delay digestion does not account for the low heat increments of diets containing fat because these increments were determined not from single feedings of the test nutrient but by difference between observations on the heat production from established states of nutrition.

In offering numerical expressions of the associative effects of nutrient combination on the economy of utilization of food energy one can say that the experimentally determined dynamic effect of carbohydrate and protein was 12.5% less than as computed from the dynamic effects of the individual nutrients; and similarly, the dynamic effect of carbohydrate and fat was 35% less, of protein and fat 54% less, and of carbohydrate, protein and fat 22% less, than as computed from the dynamic effects of the individual nutrients.

Referring again to table 5, the net energy values of the mixed supplements, as per cent of their metabolizable energy, are shown to be materially higher, especially for those containing protein, as experi-

mentally determined than as computed from the individually determined net energy values for the protein, carbohydrate and fat—thus expressing the final effects of nutritive combination on energy value.

The dynamic effects of diets, therefore, are not the additive effects of their components, and are not in accord with their protein contents; and inasmuch as there is no scientific means of apportioning energy effects or values among dietary constituents, the dynamic effects of individual foods or nutrients are without significance as constants.

While there is a question as to the applicability of results obtained with one species of animal to the nutrition of another species, the results of this study suggest, at face value, that it is not necessary to diminish the protein content of the hot-weather human diet in order to have a low heat increment, since this purpose can be accomplished by the equicaloric substitution of fat for carbohydrate.

It thus appears that there is physiological economy in the high fat content of the diet of working people of some tropical countries—notably Brazil.

That the protein contents of diets do not dominate the heat increment is shown by five series of experiments conducted at this laboratory (Forbes et al., '35, '38, '39a, '40), with mature as well as with growing rats as subjects, in which the heat production of animals receiving equicaloric diets differing in protein content decreased slightly in the order of the increase in protein.

Also the results of this study imply that manufacturing processes which decrease the fat content of by-product feeds serve to lower the net energy value of the products not only through diminishing their gross energy but also by increasing the energy expense of their utilization.

SUMMARY

A study was made, with mature albino rats as subjects, of the dynamic effects of beef protein, cerelese (corn sugar) and lard, individually and variously combined, at planes of nutrition above maintenance; the dynamic effects being measured by difference between the heat production of the subjects as established on a complete basal diet and on the same plus the nutritive supplements.

The supplementary feeding of protein, of carbohydrate or of fat increased the production of heat from the kind of nutriment fed. Cerelese spared protein and fat; beef protein spared carbohydrate and fat; while lard spared only protein.

Of the mixed supplements, cerelese and beef protein increased heat production from carbohydrate and protein, and spared fat; cerelese and lard increased heat production from carbohydrate, and spared protein and fat; beef protein and lard increased heat production from protein, and spared carbohydrate and fat; while cerelese, beef protein and lard increased heat production from protein and carbohydrate, and spared fat. All mixed supplements, regardless of composition, spared fat.

Fed as supplements to a complete diet sufficient for maintenance, the dynamic effect of beef protein was 32%, of cerelese 20%, and of lard 16% of its gross energy.

The dynamic effects of mixed supplements of protein and fat, and of carbohydrate and fat, were lower even than the dynamic effect of fat. In this sense fat is much more potent than are protein and carbohydrate in determining the dynamic effects of diets.

Among four supplemental combinations of protein, carbohydrate and fat, computed to 1000 cal. of gross energy, the one with the lowest dynamic effect was that of protein and fat, followed closely by the combination of carbohydrate and fat; then by the combination of protein, carbohydrate and fat; while the combination with the highest dynamic effect was that of carbohydrate and protein.

The observed dynamic effect of carbohydrate and protein was 12.5% less, of carbohydrate and fat 35% less, of protein and fat 54% less, and of carbohydrate, protein and fat 22% less, than as computed from experimentally determined values for the individual nutrients.

The dynamic effects of diets are not the additive dynamic effects of their components; the prevalent idea that the dynamic effects of diets vary in the order of their protein contents is incorrect; and inasmuch as there is no scientific means of apportioning energy effects or values among dietary constituents, the dynamic effects of individual foods or nutrients are without significance as constants.

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THE BIOASSAY OF VITAMIN E¹

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ONE FIGURE

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In an attempt to standardize the technique employed in the bioassay of vitamin E and thereby increase the accuracy of the test, a number of refinements have been made in the original method. Bacharach and co-workers ('37, '38) recommended the use of virgin animals. They found that rats in which sterility had been established showed more failures in implantation in the next pregnancy than did virgin animals. Bacharach ('38) also observed that a larger dose of vitamin E was required to insure fertility after a resorption gestation than with virgin rats.

Mason and Bryan ('38, '40) overcame initial storage by the feeding of a vitamin E low diet to the breeders during the latter half of the lactation period. The suckling young were thus limited in their vitamin E storage to that conferred through placental and mammary transfer. The weanlings were maintained on the vitamin E low diet; such prepared females invariably resorbed during their first gestation, thus eliminating the necessity of a trial pregnancy.

Mason ('39) studied the relation between the time of dosage and the response evoked. The best results were obtained if the test preparation was given on the eighth day of the gestation; however, if the test material was administered in several doses from the fourth to the eighth day inclusive of the pregnancy, the sensitivity of the test was further increased. Mason ('42) later reported that the state of the uterus on the sixteenth day of pregnancy was the best index of vitamin E activity. The presence of two or more viable fetuses in the uterus on the sixteenth day of pregnancy was considered a positive response.

¹ Aided by grants from the Board of Research and from the Department of Agriculture of the University of California, and the Rockefeller Foundation, New York City. The following materials were generously contributed: natural alpha-tocopherol by Merck and Company, Inc., Rahway, New Jersey; alpha-tocopheryl acetate by the League of Nations Committee on Vitamin Standardization; yeast by the Vitamin Food Company, New York; and calciferol by the Withrop Chemical Company, Rensselaer, New York.

This shortened assay period not only effected a saving of time and materials but contributed appreciably to the accuracy of the test. This technique, however, is subject to criticism in that resorption of the fetuses may be initiated late in the gestation.

Palmer ('37) evaluated vitamin E activity by reference to the percentage of total implantations resulting in living or in dead young. A live litter efficiency of 10% or more was considered a positive response.

Gottlieb, Quackenbush and Steenbock ('43) found that within certain limits females maintained on a low fat vitamin E free diet increased in weight during pregnancy in direct proportion to the quantity of vitamin E administered. For an equal response, the requirement for vitamin E on the low fat diet was less than half of that on the high fat ration. Homrich ('43) has recently reported that the use of a fat-free basal diet during the dosing period resulted in higher live litters and live implantation efficiencies than were observed with the standard lard, cod liver oil containing ration.

The studies herein reported were undertaken in an effort to increase the sensitivity of the vitamin E assay.

METHOD AND RESULTS

Virgin and resorption gestation rats were employed. The test materials were administered in single or multiple doses. Two types of diets were fed, one in which the vitamin E was destroyed by the incipient rancidity of the lard in the ration, and the other in which the vitamin E was removed by rigorous extraction of potential vitamin E containing ingredients in the diet with fat solvents. The diets used are given in table 1.

Littermate sisters were segregated at weaning so that each of the dietary groups was represented by one littermate. The rats on such dietary regimens were employed for three studies as follows: growth (the rats in this study were not bred), resorption gestation (the animals in this group were bred for a trial gestation and were used for curative assay in a subsequent pregnancy), virgin (the animals in this category were employed for cures after sterility had been established in the resorption gestation group).

Growth

The early growth rate of rats maintained on diet 427 was somewhat superior to that obtained with the other diets (fig. 1). This perhaps

TABLE 1
Diets deficient in vitamin E.

Diet 427 is made deficient by incipient rancidity of the lard. In the diets 821 A-D the vitamin E is removed by extraction with fat solvents.

DIETARY COMPONENT	DIET	
	427	821 A-D
	gm./100 gm	gm /100 gm
Casein		
(Tech.)	27	
(Alcohol ether extracted)	..	24
Cooked ground corn starch	35	..
Sucrose	..	72
Salts (McCollum 185)	4	4
Brewers' yeast	10	..
Lard	22	..
Cod liver oil	2	..

Supplements fed daily to groups receiving diets 821 A-D.

	821A	821B	821 C	821D
Carotene (S.M.A.)	80 µg.	80 µg.	80 µg.	..
Cod liver oil	40 mg.
Calciferol	12 IU	12 IU	12 IU	..
Ethyl linoleate	60 mg.
Peanut oil ¹	..	250 mg.	250 mg.	250 mg.
Brewers' yeast	1 gm.	1 gm.
Ether extracted brewers' yeast	1 gm.	1 gm.

¹ 250 mg. of the oil was equivalent in its linoleic acid content to 60 mg. of ethyl linoleate. The peanut oil employed in these studies failed to invoke fertility in rats of proved sterility, when fed to eight rats at a level of 8 gm. (2 gm. on alternate days during the first 7 days of the gestation).

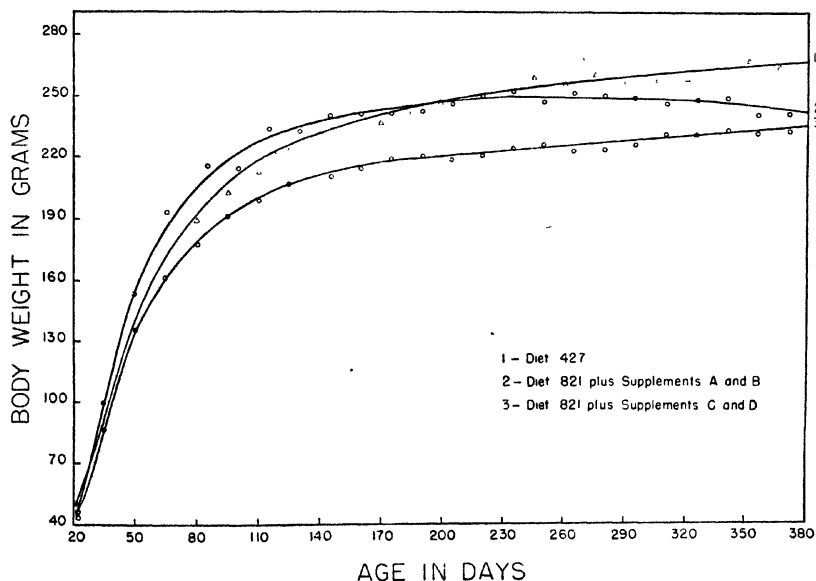


Fig. 1 Growth of female rats on the various types of E-low diets.

can be explained either by the potential growth stimulation of the fat in the ration or by the fact that the yeast and cod liver oil were incorporated in the diet while supplements were offered to the animals in the remaining groups. The rats did not consume the vitamins in their entirety for a period of a fortnight or longer. Slightly better growth was observed with rats receiving the unextracted than the extracted yeast.

First litter fertility

Eight to ten rats in the several dietary groups were bred for their trial gestation at the first pro-oestrus after 60 days. These rats were mated in late summer, a time of year in which first litter fertility was known to occur. As can be seen from table 2, the highest incidence of first litter fertility was observed with the rats receiving the lard, cod liver oil containing ration (diet 427). These rats resorbed in a subsequent gestation.

TABLE 2

Incidence of first litter fertility in rats reared on vitamin E-low diets.

DIET	NUMBER OF RATS	NUMBER OF RESORPTIONS	NUMBER OF LITTERS	AVERAGE NUMBER OF YOUNG PER LITTER	AVERAGE WEIGHT IN GRAMS
427	10	2	8 ¹	7.8 ²	6.1
821A	9	8	1	1.0 (dead)	5.0
821B	10	8	2	3.5	5.0
821C	8	7	1	8.0	5.8
821D	9	7	2	4.0	4.6

¹ Two were questionable; loss of weight indicated that small litters were cast.

² Average of 6.

Curative assays with single doses

A single dose of a 3 mg. of alpha-tocopherol was administered on the first day of the gestation. Resorptions (table 3) resulted only with those rats receiving the cod liver oil or lard and cod liver oil in their rations (see diets 427 and 821D). The response to the single dose of the tocopherol was essentially the same for the virgin and for the resorption gestation groups. It is of interest to note that in late life, muscular dystrophy was observed only in rats receiving diet 427 despite the fact that the first litter fertility of early adulthood indicated a greater storage of vitamin E than in the other dietary groups.

As judged by litter quality,² the young cast by mothers receiving diets without cod liver oil were slightly superior to those of the

² The number of young per litter, their average weight in grams, the percentage of dead young in the litter, and the appearance of the live young.

mothers maintained on the cod liver oil containing rations; therefore, the use of carotene and calciferol is indicated.

Virgin rats and rats of proved sterility served equally well as test animals. In our opinion, the latter are preferable as the need for controls is obviated.

TABLE 3

The vitamin E activity of a single dose of 3 mg. of alpha-tocopherol administered to virgin and resorption gestation rats maintained on vitamin E-low diets.

DIET AND GROUP	NO. OF RATS	NO. OF IMPLAN-TATIONS	NO. OF RESORP-TIONS	% OF IMPLAN-TATIONS LITTERING	NO. OF LITTERS WITH LVG. YOUNG	AV. NO. OF LVG. YOUNG PER LITTER	AV. WT. IN GMS.	LITTERS OF DEAD YOUNG
427								
Virgin	10	10	1	90	6	5.6	4.6	3
Resorption	7	7	1	86	5	6.8	4.3	1
Total	17	17	2	88	11	6.1	4.5	4
821A								
Virgin	10	10	0	100	10	6.6	5.2	0
Resorption	8	8	0	100	8	7.1	5.4	0
Total	18	18	0	100	18	6.8	5.3	0
821B								
Virgin	10	10	0	100	9	7.7	5.5	1
Resorption	9	9	0	100	9	7.7	5.7	0
Total	19	19	0	100	18	7.7	5.6	1
821C								
Virgin	10	10	0	100	10	6.7	5.5	0
Resorption	7	7	0	100	7	8.1	5.9	0
Total	17	17	0	100	17	7.3	5.8	0
821D								
Virgin	10	9	1	89	8	6.6	5.7	0
Resorption	8	8	1	88	7	6.6	5.5	0
Total	18	17	2	88	15	6.6	5.6	0

Curative assays — divided and single doses — diet 427

A comparison was made of the response to multiple dosing with several levels of alpha-tocopheryl acetate of virgin and resorption gestation rats maintained on the standard diet 427. Positive controls received 3 mg. of tocopherol as a single dose or in divided dosage.

The alpha-tocopheryl acetate fed in these experiments was supplied by the League of Nations Committee on Vitamin Standardization. The

TABLE 4

The vitamin E activity of several levels of alpha-tocopheryl acetate with virgin and resorption gestation rats maintained on diet 427.

GROUP	NO. OF RATS	NO. OF IMPLAN-TATIONS	NO. OF RESORP-TIONS	% OF IMPLAN-TATIONS LITTERING	NO. OF LITTERS WITH LVG. YOUNG	AV. NO. OF LVG. YOUNG PER LITTER	AV. WT. IN GMS.	LITTERS OF DEAD YOUNG
Divided dose 0.8 mg. alpha-toco. Ac.								
Virgin	20	20	17	15	1	8.0	4.0	2
Resorption	13	12	10	17	2	3.3	3.3	..
Total	33	32	27	16	3	4.8	3.7	2
1.2 mg. alpha-toco. Ac.								
Virgin	19	19	9	53	10	4.0	4.3	..
Resorption	12	12	5	59	7	4.6	4.1	..
Total	31	31	14	55	17	4.3	4.2	..
1.8 mg. alpha-toco. Ac.								
Virgin	20	20	2	90	17	7.4	4.9	1
Resorption	13	13	1 *	92	9	6.1	4.5	3
Total	33	33	3	91	26	7.0	4.8	4
2.7 mg. alpha-toco. Ac.								
Virgin	20	20	0	100	20	8.6	5.2	..
Resorption	13	13	1 *	92	7	8.3	5.0	5
Total	33	33	1	97	27	8.5	5.2	5
3.0 mg. alpha-toco.								
Virgin	10	9	0	100	9	8.7	5.7	..
Resorption	11	11	0	100	11	7.9	5.8	..
Total	21	20	0	100	20	8.3	5.8	..
Single dose 3.0 mg. alpha-toco.								
Virgin	10	10	2	80	8	8.9	6.0	..
Resorption	15	14	1	93	11	7.0	5.7	2
Total	25	24	3	88	19	7.8	5.8	2

* Late.

test solutions were diluted with olive oil so that the total dose for one rat was contained in 0.5 ml. The single dose contained in 0.5 ml. of olive oil was fed on the first day of the gestation (as detected by the presence of sperm in the vaginal canal), the divided dose as 0.1 ml. on each of the first 5 days of the gestation. Negative controls received only the solvent; positive controls 3 mg. of natural alpha-tocopherol. The animals destined to receive each sample were selected over a 1- or 2-day period; thus each dilution was used for 5 or 6 days. The solutions were kept at 2°C. and saturated with CO₂ when not in use.

Early resorption gestations occurred in the negative control group indicating that rats maintained on diet 427 from day 21 and starting a gestation at 110 days were sterile although first litter fertility may ensue if the gestation is initiated at 60 days.

Significant differences in the percentages littering in the virgin as compared with the resorption gestation animals were not observed, but the litter quality was somewhat superior in the virgin group. The results for the virgin and the resorption gestation groups are tabulated both separately and combined (table 4).

The minimal effective dose to insure fertility appeared to lie between 1.8 and 2.7 mg. of alpha-tocopheryl acetate. The divided dose had a slight advantage over the single dose.

DISCUSSION

The low fat cod liver oil free rations were superior to the standard high fat diet. The incidence of first litter fertility was materially decreased by the feeding of the low fat ration; the administration of a single dose of 3 mg. of alpha-tocopherol produced a somewhat higher litter response with rats receiving the extracted diet than with the high fat rations. The superiority of the low fat type of diet is in agreement with the findings of Gottlieb et al. ('43) and Homrich ('43).

The virgin rat afforded little advantage over the animal of proved fertility. The failures in implantation reported by Bacharach and co-workers ('37, '38) were encountered only occasionally in either the virgin or the resorption gestation groups.

The response of a group of animals maintained on the standard diet (427) to a single dose of alpha-tocopheryl acetate indicated that the most sensitive response was obtained when the test material was administered over a period of several days. Litter quality paralleled the percentage littering.

SUMMARY

A comparison was made of the standard lard cod liver oil containing diet with rations composed of vitamin E free ingredients.

During the period of early growth the rats receiving the high fat ration grew at a more rapid rate than did their sisters maintained on the low fat diets, but after several months significant differences were not apparent. The rats receiving a diet supplemented with brewers' yeast attained a slightly higher weight plateau than did the rats receiving extracted brewers' yeast.

The highest incidence of first litter fertility was observed with rats maintained on the high fat diet; nevertheless, the rats maintained on this ration were less sensitive as test animals for vitamin E assays than were rats raised on the extracted diets. Unextracted yeast can be employed to advantage in vitamin E low rations. Peanut oil may replace ethyl linoleate as a vitamin E free source of the essential fatty acids.

Multiple, as contrasted with single dosage of alpha-tocopherol, appeared to result in a higher percentage of litters.

Virgin and resorption gestation rats served equally well as test animals. First litter fertility was encountered if the trial gestations were initiated at 60 days, but not at 110 days.

The minimal effective level of alpha-tocopheryl acetate to insure fertility in rats maintained on the high fat standard ration is between 1.8 and 2.7 mg.

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CONGENITAL MALFORMATIONS INDUCED IN RATS BY MATERNAL NUTRITIONAL DEFICIENCY

VI. THE PREVENTIVE FACTOR¹

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Congenital malformations have been described in the offspring of female rats reared and bred on a diet of the following percentage composition: yellow corn meal 76, wheat gluten 20, calcium carbonate (C.P.) 3 and sodium chloride (C.P.) 1. This diet supplemented every tenth day by 60 I.U. of vitamin D as viosterol was called diet I. The deformed offspring showed shortening of the tibia, mandible, fibula, radius and ulna, fusion of ribs, fingers, and toes, and cleft palate. While there were quantitative differences of the malformations in different specimens, they all conformed to a definite pattern, the "pattern of diet I" (Warkany and Nelson, '40, '41 and Warkany, Nelson and Schraffenberger, '43a, '43b). Identical malformations were obtained in the offspring of rats of the Sprague-Dawley strain (albino), Long-Evans strain (hooded) and a strain of albino and hooded rats obtained from the Pediatric Department of Johns Hopkins University. It became obvious that a nutritional phenomenon was being dealt with since females of the same strains did not produce offspring of the pattern of diet I when they were bred while receiving an adequate stock diet. It was also found that the addition of 2% pig liver to the maternal diet I prevented the appearance of these abnormalities. When females, which had produced abnormal offspring while on diet I, were given diet I supplemented with liver before they were mated again, they always produced anatomically normal young. This led to the conclusion that a dietary factor deficient in diet I and present in large amounts in pig liver was necessary for the normal prenatal development of the rat. The search for this preventive factor will be described in the following pages. An inquiry into a maternal nutritional deficiency which

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manifests itself in the offspring differs from other investigations concerned with the finding of a vitamin or a vitamin-like factor. The stores of the maternal tissues act as "buffers" which prevent deprivation of the developing embryo as long as possible. In fact, it was assumed until recently that these maternal stores either protect the offspring completely, thus resulting in the delivery of normal young, or that in case of extreme dietary deficiency the embryos die in utero. Although there is some truth in this "all or none" theory, it is not entirely correct. Between the two there exists a narrow range in which maternal nutritional deficiency may result in arrest of the embryos' development without causing death. In this case congenitally deformed offspring may be the result.

The breeding of animals fed the deficient diet I gave results which conformed to this concept. Certain difficulties became apparent in the search for the maternal dietary factor capable of preventing malformations in the offspring. The females reared and mated while fed diet I either had no issue at all, or they produced normal young, or they had — comparatively rarely — deformed offspring. Under the most favorable circumstances only one-third of the young were anatomically defective. Abnormal offspring were less often the result of first than of second and later pregnancies. Thus, in order to demonstrate the preventive power of a supplement to diet I, it was necessary to obtain a large number of normal young from females that had been bred repeatedly. We considered a supplement to be preventive when more than 300 young, the results of first, second and third litters were found anatomically normal on external inspection and after clearing with the Schultze-Dawson method. On the other hand, the appearance of a very few abnormal young on a supplemented diet provided strong evidence that the supplement was not preventive.

We reported in previous communications (Warkany and Nelson, '42a; Warkany, Nelson and Schraffenberger, '42) that supplements of liver and alcoholic liver extract were preventive, while supplements of iodine, manganese, casein, alfalfa leaf meal, and cod liver oil were not preventive. Since then we have found that a supplement of 4% of liver ash is not preventive. Wheat germ oil (14 drops weekly administered by pipette) as a supplement to diet I did not inhibit the appearance of abnormalities. Since liver contains many vitamins of the B-complex in large quantities, we proceeded to test several crystalline substances of this group.

Thirty-eight females were fed diet I supplemented by 5 mg. each of riboflavin, thiamine hydrochloride, niacin, calcium pantothenate and

pyridoxine hydrochloride per 100 gm. of the diet. From these 38 females 55 litters consisting of a total of 371 young were obtained. Two hundred and thirty-three offspring were the result of first, 101 of second, 19 of third and 18 of fourth pregnancies. All 371 young were anatomically normal. It appeared, therefore, that the combination of the 5 crystalline vitamins mentioned prevented the abnormalities of the pattern of diet I. Another group fed diet I supplemented by thiamine hydrochloride, niacin, calcium pantothenate, and pyridoxine hydrochloride in the same quantities, produced a number of abnormal offspring (table 1). A supplement of pyridoxine hydrochloride plus calcium pantothenate failed to prevent the abnormalities. Thiamine hydrochloride alone as well as niacin alone were insufficient supplements, while riboflavin alone

TABLE 1

The effect of vitamin supplementation of diet I on the appearance of congenital malformations.

MOTHERS FED DIET I SUPPLEMENTED BY	OFFSPRING		
	Total	Normal	Abnormal
Riboflavin + thiamine + niacin + pyridoxine + Ca pantothenate	371	371	0
Thiamine + niacin + pyridoxine + Ca pantothenate	51	27	24
Pyridoxine + Ca pantothenate	67	47	20
Thiamine	152	125	27
Niacin	180	161	19
Riboflavin	319	319	0

proved preventive. In the group receiving diet I plus 5 mg. of riboflavin per 100 gm. of diet, 36 females produced 55 litters consisting of 319 anatomically normal offspring. Of these young 211 were the result of first, 100 of second, and 8 of third pregnancies. These experiments seemed to indicate that riboflavin is the preventive factor sought for.

The crucial experiment remained to be done, namely, the production and prevention of the malformations on highly purified diets in which most of the nutritional factors were of known chemical constitution and in which the vitamin B-complex was represented by crystalline preparations only. It has been briefly reported (Warkany and Schraffenberger, '43) that female rats on a purified diet lacking riboflavin produced a small number of young with malformations which were identical with those of the pattern of diet I. The diet used for this purpose had the following percentage composition: sucrose 68, vitamin

test casein 18, vegetable oil 10,² and salt mixture 4.³ One hundred grams of this diet were supplemented by 0.8 mg. thiamine hydrochloride, 0.8 mg. pyridoxine hydrochloride, 1 mg. calcium pantothenate, 10 mg. niacinamide⁴ and 100 mg. choline chloride.⁵ Every tenth day 1 to 2 drops of a 1:4 dilution with olive oil of "Drisdol with Vitamin A in Oil"⁶ were administered by pipette.

We have repeated these experiments and improved the yield of abnormal offspring by keeping the females on the riboflavin deficient diet for about 3 weeks before breeding. The diet described above was also somewhat modified in that it was supplemented every tenth day by vitamin E (1 drop of a solution of 5 gm. alpha tocopherol in 100 ml. of olive oil), vitamin K (1 drop of a solution of 1 mg. of 2-Methyl-Naphthoquinone in 5 ml. of olive oil) and vitamins A and D (2 drops of a 1:4 solution of "Drisdol with Vitamin A in Oil"). This supplemented purified diet without riboflavin will hereafter be called diet Z. The females, which were about 3 months old and weighed about 200 gm. were put on diet Z for 3 weeks after which, whenever they were in estrus, they were bred to males which had been kept on an adequate stock diet. Some of the females became pregnant immediately, others only after several matings and a third group became sterile before they were impregnated. Of 54 females of this group 25 have had litters up to the present time. There were 25 first and only one second litter. These litters consisted of 137 young of which 104 were anatomically normal and 33 were abnormal. The abnormalities were entirely identical with those of the pattern of diet I.

Thirty-four females were fed diet Z supplemented by 0.8 mg. riboflavin per 100 gm. of diet. Twenty-seven of these females had litters. There were 27 first, 2 second and 1 third litters. These litters consisted of 120 anatomically normal young (table 2).

These experiments with purified diets are in accord with the assumption that a deficiency of riboflavin in the maternal diet is responsible for the abnormalities of the pattern of diet I in a part of the offspring.

About $\frac{1}{2}$ of the females fed diet Z for several weeks stopped having estrus cycles, lost considerable weight and showed signs of "malnutrition". An attempt was made to "restore" these animals by giving them small amounts of riboflavin by pipette. It has been our experience that rats are unable to reproduce on diet Z when their weight falls

¹ The mixture of sucrose, casein and vegetable oil was furnished by S.M.A. Corporation.

² Salt mixture no. 351 as recommended by Hubbell, Mendel and Wakeman ('37).

³ Merck.

⁴ SMACO.

⁵ Winthrop.

to 175 gm. or below. To such animals we gave 4 drops of an aqueous solution of crystalline riboflavin (12 mg. of riboflavin in 100 ml. of distilled water). As a rule this treatment resulted in a gain of weight within 24 hours. After 2 or 3 days there was usually no further increase and there might be loss of weight. Then another 4 drops of the riboflavin solution was given. This process of "titration" was continued until the animals resumed estrus cycles and regained a weight of about 200 gm. Then these restored females were bred. Up to the present time litters have been obtained from 7 of these females. In 4 of them the addition of riboflavin was discontinued after they conceived, while 3 females received 4 to 16 drops of the riboflavin solution during pregnancy. From these restored females 7 litters consisting of 52 young were obtained. Twenty-eight of these young or 53.9%, were

TABLE 2

Breeding results on diet Z and on diet Z plus riboflavin.

MOTHERS FED	OFFSPRING		
	Total	Normal	Abnormal
Diet Z	137	104	33
Diet Z + riboflavin	120	120	0

abnormal. This, in our experience, is the highest incidence of abnormalities obtained in any group of rats bred on a deficient diet. The procedure of depletion and controlled restoration of riboflavin may represent the best method of obtaining female rats which possess just enough riboflavin for reproduction but not enough for the normal development of the young. However, not all depleted animals can be restored by riboflavin. After a period of more than 4 months on diet Z, attempts at restoration have failed.

COMMENT

Various authors have attributed congenital malformations to a maternal nutritional deficiency. Zilva, Golding, Drummond and Coward ('21) observed that sows fed a diet deficient in vitamin A produced pigs in which some limbs were completely missing. Hughes ('34) reported that a purebred Poland China gilt fed a diet deficient in vitamins A and G produced six pigs, three of which were deformed. Converse and Meigs ('31) observed congenital blindness in the offspring of cows fed overripe timothy hay which had a low vitamin A content. Moore and co-workers ('35) found congenital blindness in calves, caused

by constriction of the optic nerve. They attributed the disorder to a nutritional deficiency of the dams which had received rations with a poor quality roughage. Hale ('33) described pigs without eyeballs born of a Duroc-Jersey gilt which had received a ration deficient in vitamin A. This author deserves credit for demonstrating that the defect was not genetically determined. In later publications Hale ('35, '37) described harelip, cleft palate, accessory ears and misplaced kidneys in pigs born to sows fed a vitamin A deficient diet. Several reports deal with embryonic defects in fowl which can be attributed to maternal nutritional deficiency. Micromelia and shortness of the antero-posterior axis of the skull were found by Byerly and co-workers ('35) in embryos of hens fed a deficient diet. Addition of wheat germ, liver and whey to the maternal diet prevented the abnormalities. Lyons and Insko ('37) observed chondrodystrophy in the chick embryo produced by manganese deficiency in the diet of the hen. Lepkovsky and co-workers ('38) reported that riboflavin deficiency in the breeding hen's diet produced defects such as degeneration of the wolffian bodies and reduced size of the embryo associated with death.

The experiments reported in the present communication add further proof to the thesis that maternal dietary deficiency may lead to congenital defects in the offspring. Since the malformations of the pattern of diet I could also be induced by a highly purified maternal diet without riboflavin (diet Z) and prevented by the addition of riboflavin to diet I or diet Z, it is concluded that the abnormalities of this pattern are caused by a prenatal riboflavin deficiency. This conclusion is in agreement with the fact that diet I is poor and liver rich in riboflavin. It may seem surprising that a supplement of 2% of alfalfa leaf meal to diet I did not prevent the abnormalities, since alfalfa is considered a good source of riboflavin. However, the riboflavin content of alfalfa plants is reduced to a great extent by maturation and curing (Hunt and Bethke, '40) so that a supplement of 2% may be insufficient for a pregnant rat.

We are dealing in these experiments with a borderline deficiency. The mothers apparently have sufficient riboflavin for maintenance of the estrus cycles and for gestation. The fetuses have sufficient riboflavin for growth but not for differentiation. It can be postulated that abnormal offspring appear when the riboflavin of the blood reaches a certain critical level. A reduction of riboflavin below the critical level leads to sterility or embryonic death, while an increase beyond this level results in the birth of normal young. It is understandable that such a borderline deficiency may lead to the production of normal, abnormal and even mixed litters. Slight variations of the fetal growth

rate, of the implantation sites or of the riboflavin level may decide whether the development of the fetus takes a normal or an abnormal course. Even under strict dietary control, many difficulties are encountered in attempts to obtain abnormal young. Synthesis of riboflavin by intestinal microorganisms and coprophagy may influence the maternal riboflavin reserves. Furthermore the mothers often resorb abnormal young in utero or eat them after birth, if they are not removed immediately.

The abnormalities of the pattern of diet I are most conspicuous in the skeleton. The attempt has been made to explain the pathogenesis of these malformations on the basis of histologic (Warkany and Nelson, '42b) and experimental findings (Warkany, Nelson and Schraffenberger, '42). It can be assumed that the formation of the membranous skeleton, which precedes the cartilaginous as well as the osseous skeleton, is inhibited by the riboflavin deficiency. That a general nutritional deficiency affects certain parts of the skeleton while others are spared may not seem plausible at first. However, similar differential susceptibility of developing organisms to toxic agents is well known (Child, '41). Our experiments show that such a differential susceptibility exists also to nutritional deficiency. This differential behavior is not obvious in the cephalocaudal direction, since bones of the head as well as those of the axial and appendicular skeleton are affected. However, in the transverse plane we note a differential sensitiveness, since in general the skeletal structures near the mid-dorsal region are normal while the more ventro-distally situated ones are more often deformed.

Gross malformations of soft tissues have been observed occasionally in the abnormal young of the pattern of diet I. Encephalocele, "open eye" (a severe ocular deformity), gastroschisis, reduction in size of the urogenital papilla and spina bifida were seen in rare specimens. We have not felt justified in including these rare defects in the pattern of diet I. However, thorough studies of the soft tissues have not yet been made. Such studies may reveal that the formation of the skeleton is not the only developmental process adversely affected by prenatal riboflavin deficiency.

SUMMARY

1. The congenital malformations of the pattern of diet I are prevented when the maternal diet I is supplemented by riboflavin.
2. Supplements of thiamine hydrochloride, niacin, pyridoxine and calcium pantothenate are not preventive.
3. On a purified maternal diet in which the vitamin B-complex is represented by crystalline substances, malformations of the pattern of diet I appear in the offspring when riboflavin is omitted.

4. On the same diet supplemented by sufficient riboflavin no deformed offspring are observed.

5. It is concluded that a deficiency of the maternal diet in riboflavin is responsible for the congenital malformations of the pattern of diet I.

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ABSENCE OF RAPID DETERIORATION IN MEN DOING HARD PHYSICAL WORK ON A RESTRICTED INTAKE OF VITAMINS OF THE B COMPLEX ¹

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It has been claimed that diets markedly deficient in vitamins of the B complex provoke serious physical and psychological deterioration in a few days of hard work (Johnson et al., '42). If true this would be of much importance. However, we believe there were serious faults in the experiment and observations. The present report covers experiments in which detailed objective data were obtained under rigidly controlled conditions. The dietary limitations and the intensity of work were similar to those applied in the work of Johnson et al. ('42), parallel control subjects were studied, the period of restriction was twice as long, and "before" and "after" control periods were included.

PROGRAM

The general plan was to make frequent precise observations and measurements before, during, and after a period of hard physical work involving a daily expenditure of 4,500 to 5,000 Cal. A constant diet was used but the intake of B vitamins was varied by daily vitamin capsules or placebos so that a period of 14 days of low intake was preceded by an intake approximating National Research Council recommendations and was followed by 5 days of supplementation at a moderately high level. The responses were measured, in terms of physiological, biochemical and psychological variables, in set conditions and fixed tasks

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involving endurance, anerobic work strength, work capacity, speed and coordination.

The basal diet was very low in B vitamins but adequate in calories, protein, minerals and vitamins A, C and D. A rigid daily schedule of physical work, tests, meals and rest was followed. The subjects were divided into a control group of three men who received vitamin supplements daily and a "deficient" group of five men who received only placebos for a period of 14 days. Following the deficient period there was a period of 5 days when both groups received supplements. In this way both individual and group controls were provided.

During a preliminary period of 21 days the men were carefully standardized and control measurements and observations were made. This was followed by 14 days of "deficiency" and then 5 days of "recovery." In the last 19 days the daily intake of 4,640 Cal. was not quite sufficient to maintain caloric balance in any of the men; there was an average total loss of 2 pounds (maximum 4 pounds) in body weight. Throughout, all men rigidly followed fixed daily routines. The principal energy expenditure was caused by set tasks on the motor-driven treadmill. In the hard work period there were four forced marches daily of 60 minutes each at 3.5 miles per hour on the treadmill set at a 10% rate of climb. Pulse rates were counted at set times during and after this work. A battery of psychomotor tests was applied during work on alternate days at the start and at the end of the treadmill work. At the end of the second hour of this "forced" marching the men ran for 90 seconds at 9.0 miles per hour and a grade of 8.6% ("anerobic work"). At the end of the main work every other day additional special tests were made, such as the treadmill version of the Fatigue Laboratory Test (Johnson, Brouha and Darling, '42) and the Army Air Forces Fitness Test as used by Lt. L. A. Larson, A.A.F. The latter comprises "sit-ups," "push-ups" and a 300-yard shuttle race. Arm vein blood was drawn every fourth day during rest before and at 8, 12 and 30 minutes after the anerobic work. Every other day each man filled out a questionnaire on his subjective state. Detailed clinical examinations were made repeatedly. These involved the use of special ophthalmological, electrocardiographic and roentgenographic methods. Urine collections (24-hour) were made frequently. The subjects were men 19 to 30 years of age with no signs or symptoms of significant abnormalities. Under complete supervision they worked, slept and ate entirely within the confines of the laboratory. The experiment rooms were maintained at 77°F. and 50% relative humidity at all times. Standard gymnasium clothing was worn in all experiments.

METHODS

All clinical analyses were made in duplicate with frequent blanks, standards and controls. Blood lactate was estimated by the method of Friedemann, Cotonio and Shaffer as modified by Edwards ('38). Blood pyruvate was estimated by the method of Lu ('39) as modified by Friedemann and Haugen ('42, '43). Blood sugar was measured by the method of Folin and Wu ('20) with cadmium hydroxide as the precipitating agent. Hemoglobin was determined as oxyhemoglobin with a photoelectric colorimeter. The 24-hour urine was collected in amber glass bottles containing 5 ml. of glacial acetic acid and 3 ml. of toluol. The samples were stored at 3°C. while awaiting analysis. Thiamine was estimated by the thiochrome method of Hennessy and Cerecedo ('39) as modified by the Research Corporation Committee for the Determination of Thiamine ('41). Riboflavin in food was determined by the microbiological method of Snell and Strong ('39) and in urine by the fluorometric method of Conner and Straub ('41). Niacin in food was measured by the method of Snell and Wright ('41). The thiamine method as used here has been checked for foods against standard biological assays.

Grip and back strength were measured with standard dynamometers. These measurements, as well as the flicker fusion frequency, were made in the morning before the beginning of the treadmill work and in the afternoon after the last standard 60-minute hard work period. Manual speed and control were tested with a ball-and-pipe test, motor control by tracing a stylus through a winding path, and speed of small movements by the tapping test as used previously (Keys et al., '43). Another test made during actual work on the treadmill was a measurement of reaction time involving gross bodily movements and postural adjustments.

Diet

Four fixed menus were used in rotation for all subjects. They provided an average of 35% of calories as fat. Protein adequacy was ensured (85 gm. daily average) by the use of vitamin-free casein and gluten. Only flavoring amounts of chicken, veal or beef were allowed (20 gm. daily). All flour and cereals were low-vitamin preparations. "Cream" was prepared by diluting a special 70% butterfat cream. The principal food items were macaroni or polished rice (400 gm.), bread (270 gm.), jello (150 gm.), butter (90 gm.), jelly (150 gm.), "cream" (300 gm.), hard candy and sugar (60 gm.), cake, cookies and pie (200 gm.) and farina (140 gm.). Very small amounts of canned

grapefruit, grape juice, pears, peaches, cherries, apples, strawberries and string beans made up the balance except for seasonings and token amounts of fresh lettuce and celery. Supplements supplied to all men daily ensured adequacy of minerals and of vitamins A, C and D according to National Research Council recommendations.

Every day each subject swallowed capsules, the contents of which were unknown to them or to the observers. The capsules used for the control subjects in the "deficient" period, and for all subjects in the "recovery" period contained yeast concentrate and pure vitamins providing a daily total of 3.25 mg. of thiamine, 2 mg. of riboflavin, 15 mg. of niacin, 25.2 mg. of pyridoxine, and 0.5 mg. of calcium pantothenate. Both vitamin and placebo capsules were identically flavored and colored.

TABLE 1

Average total (food plus capsules) vitamin intakes in milligrams per 1,000 Cal., and, in brackets, milligram per day for the three control subjects and the five "deficient" subjects. Average total caloric intakes per day are also given. Note the average caloric expenditures were approximately equal to the intakes for the "Preliminary" and "Control" periods but were about 150 to 200 Cal. per day greater than the intakes in the "Deficient" and "Recovery" periods.

PERIOD	PRELIMINARY	CONTROL	DEFICIENT	RECOVERY
No. of days	15	6	14	5
Cal. intake	3,560	4,430	4,640	4,640
Thiamine (control)	0.44 (1.57)	0.85 (3.78)	0.86 (3.99)	0.86 (3.99)
Thiamine (deficient)	0.44 (1.57)	0.85 (3.78)	0.16 (0.74)	0.86 (3.99)
Riboflavin (control)	0.56 (2.00)	0.68 (3.00)	0.59 (2.74)	0.55 (2.55)
Riboflavin (deficient)	0.56 (2.00)	0.68 (3.00)	0.15 (0.70)	0.55 (2.55)
Niacin (control)	5.9 (20.9)	5.3 (23.8)	5.0 (23.2)	5.3 (24.6)
Niacin (deficient)	5.9 (20.9)	5.3 (23.8)	1.8 (8.3)	5.3 (24.6)

It should be noted that prior to the experiment all of the subjects had subsisted for some months in a civilian public service camp where the intake of B vitamins was suspected but not proved to be low. In order to standardize the body stores of these vitamins, a period of 21 days preceding the "deficient" period was devoted to standardization of both the diet and the test procedures. Effort was made to approximate the National Research Council recommendations for this period. The actual intakes minus the N.R.C. recommendations for these caloric levels averaged, in mg. per man per day, for these 21 days as follows: Thiamine = + 0.14, riboflavin = - 0.72, niacin = + 1.0.

All meals were prepared, weighed out and eaten under the supervision of a dietitian. Identical meals were analyzed for vitamins and stored at - 25°C. for repeat analysis. The total daily vitamin intakes are summarized in table 1.

RESULTS

Clinical and subjective reports

No clinical changes occurred in any of the men at any time. All of the subjects were somewhat stiff and sore for the first 4 or 5 days of the hard work; this quickly disappeared and was not more prominent in the deficient than in the control group. Throughout the entire work period all subjects were tired at the end of each day as would be expected from the severity of the work. However, all of the men showed some training improvement. These phenomena were equally prominent in both groups. No anorexia, constipation, excessive fatigue, unusual muscle soreness, weakness, photophobia, cheilosis, depression, anxiety, irritability or sleeplessness occurred. The spirit was excellent at all times. The absence of subjective alterations associated with the level of the vitamin intake was clearly shown in the questionnaires. No changes were seen in electrocardiographic, x-ray or ophthalmological examinations.

Urinary excretion

Average urinary excretions of thiamine and of riboflavin for the two groups are given in table 2. The 24-hour output of thiamine clearly reflected the supplementation in the control group and remained at a

TABLE 2

Average 24-hour urinary excretion of thiamine and of riboflavin as per cent of the total intake of these vitamins. Column headings give the number of days from the start of the experiment. Compare with table 1.

PERIOD	PRE-LIMINARY	CONTROL	DEFICIENT			RECOVERY
Vitamin	1-12	19-20	25-26	29-30	33-34	40-41
Thiamine (control)	14	16	17	17	17	24
Thiamine (deficient)	15	14	14	7	5	14
Riboflavin (control)	27	27	26	26	35	37
Riboflavin (deficient)	28	29	13	26	29	28

fairly constant fraction of the intake in that group until the supplementation was stopped. The deficient group started at much the same level but showed a rapid decline in both the absolute and percentage thiamine excretions. The excretion of riboflavin was similar to that of thiamine in the control group. In the deficient group, however, the excretion as per cent of intake did not decline though the absolute riboflavin excretion quickly fell to a low level.

Since the daily vitamin intake was accurately determined, each 24-hour urinary excretion measurement constituted in itself a "satura-

tion" test. In addition, however, an extra 1.0 mg. of thiamine and 1.0 mg. of riboflavin were administered for "saturation" tests on the last day of the "deficient" diet, May 20th. The result for thiamine was an average recovery of 17.3% in the urine of the control group and 3.3% in the "deficient" group. For riboflavin the recovery averaged 37% in the control group whereas it was only from 1 to 6% in the "deficient" group.

Results of "fitness" tests

The grand averages for fitness, strength and psychomotor tests are summarized in table 3. In most of the tests, notably the Fatigue Laboratory Fitness test, all men showed some training improvement for several weeks but there was no significant differentiation between the two groups of men in any of the tests. The detailed records of the individuals offer no exceptions; i.e., as in the clinical and all other observations not a single man showed any changes which could be linked to the vitamin intake.

TABLE 3

Grand averages for the principal fitness, strength and psychomotor tests. "Preliminary Period" is the average for the tests in the last 5 days just prior to the deficiency period. "Deficiency Period" is the average for the tests in the last 6 days of the deficiency period. "Recovery Period" is the average for tests in the last 2 days of the "recovery" with supplements for all.

TEST ¹	PRELIMINARY PERIOD		DEFICIENT PERIOD		RECOVERY PERIOD	
	Control	Deficient	Control	Deficient	Control	Deficient
1. Anerobic, 1	178	172	171	168	171	172
2. Anerobic, 2	138	135	124	113	123	111
3. Endurance	133	115	118	108	123	112
4. Capacity	72	66	87	86	88	87
5. A.A.F. test	50	42	52	47
6. Grip strength	50	57	48	60	49	59
7. Back strength	305	333	328	325	358	347
8. Coordination	54	44	47	42	41	40
9. Speed — accuracy	64	62	67	64	67	68
10. Motor speed	66	67	65	63	66	63
11. Reaction speed	48	49	43	46	46	46
12. Flicker F. F.	59	60	58	58	61	59

¹ Test item no. 1 = pulse rate, in beats per minute, in the first 15 seconds after anerobic work; item no. 2 = pulse rate during 60 to 75 seconds after anerobic work; item no. 3 = average pulse during the last 5 minutes of 60 minutes of forced marching on the treadmill; item no. 4 = Fatigue Laboratory Test score; item no. 5 = A.A.F. Test score; item no. 6 = grip strength in kilograms; item no. 7 = back strength in pounds; item no. 8 = number of errors in the pattern tracing test; item no. 9 = ball-pipe circuits completed; item no. 10 = number of alternate plate taps in the first 10 seconds of tapping; item no. 11 = average gross body-posture reaction time, in units of 1/120 second, during work on the treadmill; item no. 12 = maximum flicker fusion frequency in flickers per second. Note that better performance is indicated by low scores in tests 1, 2, 3, 8, and 11.

Blood lactate and pyruvate

None of the blood lactate or pyruvate results could be interpreted as showing any relation to the level of vitamin intake. Ordinarily the most significant values are those during rest and at 12 minutes after anerobic work. The grand averages for these are summarized in table 4.

TABLE 4

Grand average values for blood lactate and pyruvate, in milligram of the acids per 100 cc. of blood, at rest and at 12 minutes after standard anerobic work. "Prelim." and "Recov." as in table 3. Other Column headings refer to the number of days on the deficient regime.

ITEM	PRELIMINARY		4TH DAY		8TH DAY		13TH DAY		RECOVERY	
	Con-trol	Defi-cient	Con-trol	Defi-cient	Con-trol	Defi-cient	Con-trol	Defi-cient	Con-trol	Defi-cient
Pyruvate, rest	0.9	0.9	0.9	0.9	0.8	1.0	1.0	1.0	0.9	1.0
Pyruvate, 12 min.	3.1	4.1	3.2	3.9	3.1	4.0	3.0	4.1	3.1	4.2
Lactate, rest	11	9	10	8	6	7	8	5	7	6
Lactate, 12 min.	72	96	55	74	41	62	50	63	55	62

DISCUSSION

The absence of essential data in the report by Johnson et al. (op. cit.) prevents exact comparison with the present results. In their study the energy expenditure, caloric intake, proportion of calories as fat, and the vitamin intake were unknown. However, proceeding from their food lists and their impressions of the level of energy output all reasonable assumptions lead to the same general rough estimates: their diet supplied, per 1,000 Cal., something like 0.12 mg. of thiamine, at least 0.25 mg. of riboflavin and perhaps 1.5 mg. of niacin. There is no basis for even this rough comparison for the other B vitamins but any difference between the two diets must have been small. The thiamine excretion of our subjects was apparently greater than that of the Harvard group but the method used by the latter for the determination must be questioned. It was reported that their "thiamine" subjects, who received 2 mg. of pure thiamine daily in addition to that in the diet, excreted an average of 200 μ g. per day. This only amounts to about 8% of the intake which is widely at variance with all other experience at this level of intake.

The subjective symptoms described by Johnson et al. ('42), are of the vague type commonly related by the neurotic patient and easily induced by suggestion. They resemble those reported by Jolliffe et al.

('39). The latter study has been effectively criticized by Wang and Yudkin ('40).

The principal evidence offered by Johnson et al. ('42), for deterioration on their restricted diet is contained in their graph on p. 591. From this it appears that a marked loss in physical fitness occurred in 2 days and that up to the time yeast was given there was only a trivial difference at most between the men who received thiamine supplements and those who did not. The most consistent difference between the two groups appeared when they were all on exactly the same regime (the yeast period). The absence of proper controls is unfortunate.

The most reasonable explanation for the results reported by Johnson et al. ('42), is provided by a consideration of the subjects and the conditions of their experiment. A group of sedentary laboratory workers, highly conditioned to expect marked effects, were suddenly put to unaccustomed severe physical work and were fed a peculiar and monotonous diet. Apparently they all suffered in differing degrees from the symptoms which have been widely publicized for thiamine deficiency, even though half the men were given thiamine supplements. One man developed "cheilosis of marked degree," though the authors themselves consider that there was no riboflavin deficiency. The physical "fitness" test used is one which is readily affected by motivation and by the stiff and sore muscles resulting from excessive unaccustomed exercise. We have also seen sudden marked improvement in the score in this test after some days of hard training.

In previous work in this laboratory we have studied the relation between physical "fitness" and performance to vitamin "supercharging" (Keys and Henschel, '42) and to restriction in thiamine and riboflavin intake (Keys, Henschel, Mickelsen and Brozek, '43; Keys, Henschel, Michelsen, Brozek and Crawford, '44). We believe that the relation between "fitness" and vitamin intake is much less close than is popularly supposed to be the case (Keys, '43; Henschel, '43). In the present study we have attempted to maintain the most rigid controls and to apply the most sensitive objective measurements to various aspects of physical "fitness." The results justify very definite conclusions as to the absence of important effects in young men on the present restriction in B vitamins during several weeks of very hard work. This does not mean that we consider the vitamin intakes used here to be adequate for more than limited periods of time.

SUMMARY

1. Eight normal young men were maintained on a rigidly controlled regime of diet, physical work and exhaustive tests for 40 days. Twenty-one days were devoted to standardization with an average intake of B vitamins approximating the National Research Council recommendations. For the next 14 days the basal diet was constant and provided an average, in milligrams per 1,000 Cal., of 0.16 of thiamine, 0.15 of riboflavin and 1.8 of niacin. During these 14 days of "restriction" all men received daily capsules. Five men received placebos; each of the other three men received abundant daily supplements of yeast concentrate and synthetic B vitamins. During the last 5 days the same basal diet was used but all eight men received these supplements.

2. None of the men knew the contents of the capsules ingested. None of the observers knew which men were receiving supplements and which only placebos. All food was directly analyzed for B vitamins as eaten.

3. The energy level was carefully standardized by a set regimen of fixed tasks, most of them on motor-driven treadmills. Balance was maintained at 3,560 Cal. daily for the first 15 days and at 4,430 Cal. for the next 6 days. Thereafter (19 days) the average intake was 4,640 Cal. and the energy expenditure about 4,800 Cal. daily.

4. Comprehensive clinical examinations, including electrocardiography and special ophthalmological details, were made at the start and at the end. A fixed schedule of repeated carefully standardized tests was maintained throughout. These included twelve objective tests covering endurance, anerobic work, speed, coordination and muscle strength. Blood lactate and pyruvate were repeatedly measured at rest and at fixed intervals after standard exhausting anerobic work. The urinary excretions of thiamine and of riboflavin were measured every few days. Psychological questionnaires were filled out by each man every other day.

5. All results were in conclusive agreement that the vitamin intake and limitation were without effect on all the functions measured. Of all the variables measured and observed only the vitamin excretion in the urine reflected the intake.

6. Claims that normal men undergo physical deterioration in a few days of hard work on diets similarly restricted in B vitamins are seriously questioned. The results reported previously are explained on the basis of factors independent of the vitamin intake, namely, faulty experiments and lack of objective measurements and proper controls.

ACKNOWLEDGMENT

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ADDENDUM

Dr. Keys has kindly shown us this paper in which he reports results diametrically opposite to those we obtained 2 years ago in a study which was similar in general plan, and he has offered us this opportunity to discuss the discrepancies. The completeness and thoroughness of Dr. Keys' work leave no doubt about the reliability of his results. Therefore one of two conclusions must be drawn: (1) either our experiments contained erroneous observations or conclusions, or both; or (2) the experimental conditions were sufficiently different to account for the difference in results.

Dr. Keys' chief criticisms are that our subjects were (1) sedentary, (2) suggestible, (3) unaffected by thiamine, and (4) that the analytical methods were poor. These criticisms, although perhaps not fully answered in the published work, were nonetheless covered in the complete protocols of our experiments. 1. Our subjects, as is customary among the workers in our laboratory, were experienced in outdoor work and in field trips and the majority had just completed experiments involving vigorous physical training. Their physical deterioration on the diet was completely different from their experience before and since on comparable field trips. 2. In answer to the criticism that suggestion was responsible for the changes observed we can only say that none of us expected any positive results in this short time. Furthermore, the thiamine pills and placebos were dispensed exactly as in Dr. Keys' experiments without the subjects knowing their content. 3. In answer to the criticism that there was no significant difference between the thiamine and placebo groups, we must point out that symptoms in the two groups were markedly different and that these symptoms were wholly different from those observed before and more recently on field trips under strenuous conditions. 4. The criticism of our analytic method for thiamine may be answered directly. The method of Egafar and Meiklejohn has proven reliable in (a) measuring accurately thiamine added to urine, (b) giving low blanks and good check values and (c) agreeing well with biological methods in the analysis of yeast.

These answers to the criticisms coupled with the results Dr. Ivy obtained which were similar to ours lead us to believe that our observations were real and our conclusions valid in the main. It would seem therefore more profitable to look to the second of the two possibilities mentioned in our first paragraph and to explore differences in experimental conditions in the hopes of reconciling the results. The main differences were: 1. Dr. Keys' subjects lived in a temperate laboratory environment; ours were outdoors in winter. 2. His subjects exercised wholly on a treadmill; ours in various outdoor activities. 3. The dietary thiamine intake of our subjects was undoubtedly lower, as shown not only by the estimates from the diet, but also from the

more rapid drop in urinary excretion of thiamine and the lower levels reached. 4. In our experiments the change in diet was more abrupt. Other smaller differences may be important, e. g., the type of food used. The recent demonstration of the biosynthesis of thiamine by Holt and Najjar complicates the entire picture. In view of this it may be necessary to consider many factors other than the intake of preformed thiamine. It is obvious that further work is necessary to clarify the problem fully.

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DIETARY PROTEIN AND PHYSICAL FITNESS IN TEMPERATE AND HOT ENVIRONMENTS¹

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Recommendations for the daily protein intake of men who are doing hard physical work have ranged from 50 to 165 gm. The low figure is that of Chittenden ('04) and the high figure that of Rubner ('02) with intermediate estimates coming from many other authors (Lusk, '31, pp. 448 and 449). The present figure given by the National Research Council ('43) of 70 gm. daily represents a compromise. Most previous suggestions have stemmed from observations on men working in a temperate environment. For men working in hot environments recommendations are commonly made that protein intake be restricted (Rubner, '02; Lusk, '31) on the ground that its high specific dynamic action imposes an unnecessary load on the heat dissipating mechanisms of the body. However, in actual practice many groups of men in this country who are accustomed to working in the heat prefer a diet containing liberal quantities of meat. Good examples are harvesters, miners and professional baseball players.

In view of the practical and theoretical importance of the problem a study has been made of subjects subsisting on three levels of protein intake. Particular attention was paid to the quantitative measurement of the subject's physical fitness for work in temperate and in hot environments.

METHODS

The general plan of the experiment was as follows: Three healthy young men were subjects. The experiment covered four successive periods of at least 6 weeks each in the winter and spring of 1942-1943.

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The diets were nutritionally complete but varied from period to period with respect to level of protein. Observations in each period fell into three distinct categories: (a) observations on diet and nutritional state, (b) observations on physical fitness for work in both temperate and hot environments, and (c) observations on metabolism.

Diet and nutritional state

The subjects ate in a college dining hall. Total caloric intake was not restricted. Control observations were made in October and November with the subjects on their customary normal diet. From November 30th to January 11th the diet was high in first-class proteins with corresponding reduction in carbohydrate. From January 12th to March 6th the diet was sharply restricted in first-class proteins with corresponding increase in carbohydrate. During this period the diet was supplemented with 10 gm. of Fleischman's dried brewers' yeast³ (type B 90) twice a week. This was to avoid the possible ill effects of B-complex deficiency. After March 6th the subjects returned to their customary diet. Dietary intakes were calculated according to standard tables (Bridges and Mattice, '42). Specimens of urine were collected frequently over 24-hour periods for estimation of nitrogen excretion. Samples of blood were drawn periodically from the subjects before breakfast for estimation of plasma protein. Urinary nitrogen and plasma protein were estimated by the micro-Kjeldahl method of Ma and Zuazaga ('42). Fasting body weight was measured frequently.

Physical fitness

In each dietary period physical fitness for hard work was tested both under temperate and under simulated desert and tropical conditions. A fourth subject subsisting at all times on a normal diet was included in all physical fitness measurements as a control against the effects of training and acclimatization.

Temperate environment. The test of Johnson, Brouha and Darling ('42) was used to assess physical fitness at 75°F., 50% relative humidity, and 0 m.p.h. wind velocity. In this test of stamina and cardiovascular fitness the subject runs on a motor driven treadmill at 7 m.p.h. up an 8.6% grade either until he is exhausted or, if not exhausted, for 5 minutes. On the basis of duration of effort and the sum of three pulses during recovery a score in arbitrary units is calculated. A change in

³ We wish to thank Standard Brands, Inc., for the gift of this yeast.

score of about 3 or 4 points is significant. Each subject was given two such tests in each dietary period.

Hot environments. Performance in the heat was tested by prolonged marches on a treadmill. Short bursts of exhausting activity were found not to be well suited for observing responses to heat. Moist, tropical conditions were simulated at 95°F., 85% relative humidity and 5 m.p.h. wind. The subjects spent 10 to 12 hours a week in the heated room. In all tests of physical fitness in the heat the grade was 2.5%, the speed 3.5 m.p.h. and the length of performance 1 hour and 20 minutes. The maximal length of marching in 1 day was 5 hours. Salt intake was adequate at all times. Water intake was constant for each subject from experiment to experiment and was at a level which had been found to satisfy thirst at all times during the preceding normal period. Fitness was judged from terminal pulse rate and rectal temperature, equal weight being placed on each of these factors.

Metabolic observations

The effects of dietary protein level on respiratory metabolism were studied by a separate set of experiments in each dietary period under temperate (75°F., 50% relative humidity, and 0 m.p.h. wind) and tropical (95°F., 85% relative humidity, and 5 m.p.h. wind) conditions. These experiments on respiratory metabolism were distinct from those designed to test physical fitness. For each set of observations the subject came to the laboratory in a fasting state and rested while lying down for at least $\frac{1}{2}$ hour in the appropriate room. His respiratory exchange was measured while reclining, standing and marching (after reaching a steady state) on a motor-driven treadmill at 3.5 m.p.h. Expired gases were collected in a Tissot gasometer or Douglas bag, and oxygen and carbon dioxide were estimated with the Haldane machine (Haldane, '35; Peters and Van Slyke, '31). In order to make the physiological stresses on the heat dissipating mechanisms comparable, a grade of 8.6% was used in the temperate room and a grade of 2.5% in the hot humid room. Neither grade of work was sufficient to cause an appreciable rise in blood lactate. Nitrogen in the urine during the periods of reclining, standing and marching was determined as described above. After completing the morning march the subject ate a late breakfast followed by lunch at the usual time. These meals were appropriate in protein content to the particular dietary period and contained approximately $\frac{2}{3}$ of the protein intake for the day. One and a half hour after lunch the subject lay down again in the same

room as in the morning and the exact procedure of the morning was repeated. The total respiratory exchange and non-protein R.Q. were calculated according to standard methods (Lusk, '31, pp. 64-69; Lusk, '24).

RESULTS AND CONCLUSIONS

For the sake of clarity the results will be presented and discussed under the following headings: (a) observations on diet and nutritional state, (b) observations on physical fitness and (c) observations on respiratory exchange.

Observations on diet and nutritional state

Table 1 presents data on dietary intake, body weight, urinary nitrogen excretion and plasma protein concentration. The figures on dietary intake are for particular days considered to be typical of the subject

TABLE 1

Dietary intake, urinary nitrogen excretion, plasma protein and body weight as affected by type of diet.

	TYPICAL DIETARY INTAKE (PER DAY)				MEAN BODY WEIGHT	MEAN URINARY NITROGEN	MEAN PLASMA PROTEIN
	Calories	Protein	Carbohydrate	Fat			
Averages		gm.	gm.	gm.	kg.	gm./day	gm./100 ml.
Normal before	3040	105	391	117	70.1	12.9	7.2
High protein	3970	149	333	226	72.0	18.5	7.4
Low protein	3250	76	431	136	70.5	9.5	7.2
Normal after	3350	108	386	153	71.8	13.5	7.1

for the entire period under consideration. It will be noted that the average protein intake during the low protein period was 76 gm. per day, a rate of intake which would not be considered "low" by many authors. However, in view of the two following facts it may justifiably be called a "low protein diet." First, our subjects averaged over 100 gm. per day during both normal periods. Hence 76 gm. per day is a low protein diet for them. Second, our subjects were consuming over 3,000 Cal. per day, and with this caloric intake it is very difficult to reduce protein intake much below 76 gm. per day without resorting to highly artificial diets. Several points should be noted. First, urinary nitrogen excretion was highest during the high protein period and lowest during the low protein period. Second, plasma protein tended to be highest during the high protein period although its changes were slight. Third, changes in weight were small but maximal weight was attained during the high protein period.

Observations on physical fitness

Temperate environments. The figures given in table 2 are physical fitness scores derived as described above under METHODS. Each score is an average of two determinations made in the latter half of the particular period. Three points should be noted. First, subjects G.P., J.S. and Control showed a steady improvement from the start of the observations through the low protein period. This we believe to be a training effect resulting from the high level of physical activity of the subjects. Similar results have been noted in many other instances in this laboratory (Johnson, Brouha and Darling, '42). Subject J.P. reached a training plateau before the experiment started. Second, there are minor drops of 3 to 4 points in the scores of all subjects except J.S. during the normal period after. During this period as well as the latter part of the low protein period an epidemic of the common cold infected all subjects except J.S. We attribute this small decrease in

TABLE 2

Physical fitness scores in a temperate environment.

(75° F., 50% relative humidity. Each figure an average of two. Scores are in arbitrary units.)

	NORMAL BEFORE	HIGH PROTEIN	LOW PROTEIN	NORMAL AFTER
J.P.	40	40	40	37
J.S.	54	67	74	76
G.P.	85	90	93	90
Average	60	66	69	68
Control	..	68	76	72

physical fitness to these colds. Third, there were no changes in physical fitness other than those noted above either in the experimental subjects or in Control. Hence we conclude that for periods of at least 6 weeks dietary protein may be maintained at any level between 75 and 150 gm. per day without affecting physical fitness as measured in temperate environments.

These results are borne out by a field nutrition study made by this laboratory on twenty-one subjects (Darling, Johnson and Pitts, '43). They also correspond with results of other authors. Chittenden ('04) obtained similar results but gave them a different interpretation. His soldier subjects reported that they tired less easily and had more stamina on the low protein diet than on the preceding normal diet. Also the professional college athletic director in charge of training the subjects reported great improvement in their physical condition toward the end of their training period (low protein diet) as compared

with the start (normal diet). Chittenden attributed both the subjective and objective improvement to the low protein diet. It seems more reasonable to interpret his results as we have ours. That is to say, a vigorous training program such as that to which his men were subjected is almost bound to lead to increased vigor and hardihood, and any adequate diet will enable this improvement to proceed. Our work supports and extends that of Bassett, Holt and Santos ('22) who, using approximately the same levels of protein intake as we have, found no effects on physical fitness of high or low protein diets lasting for 1 week; however, we lengthened the dietary periods to 6 weeks or more and have employed a much more precise measure of physical fitness.

TABLE 3

*Pulse rates and rectal temperatures during walking in hot environments.
(Means of 4 to 9 measurements on different days.)*

SUBJECT	NORMAL BEFORE		HIGH PROTEIN		LOW PROTEIN		NORMAL AFTER	
	Pulse rate /min.	Rectal temp. (°F.)	Pulse rate /min.	Rectal temp. (°F.)	Pulse rate /min.	Rectal temp. (°F.)	Pulse rate /min.	Rectal temp. (°F.)
Hot moist environment (95°F., 85% humidity)								
GP	134	101.4	128	101.5	131	101.4	128	101.5
JP	138	102.3	134	101.6	156	102.6	162	102.8
JS	143	102.1	141	101.6	139	101.6	134	101.4
Average	138	101.9	134	101.6	142	101.8	141	101.9
Control	162	102.7	156	102.8	160	102.8
Hot dry environment (110°F., 25% humidity)								
GP	127	101.2	116	100.8	114	100.2	110	100.4
JP	146	101.7	138	101.1	128	101.0	126	101.2
JS	130	101.3	122	100.9	118	100.5	118	100.2
Average	134	101.4	125	100.9	120	100.6	118	100.6
Control	148	101.4	132	101.3	152	101.9

Hot environments. The physical fitness of the subjects for intermittent work in hot environments can be deduced from the data in table 3. Therein are listed the means of pulse rates and rectal temperatures measured at comparable times in experiments performed as described above under METHODS. It will be worthwhile at this point to clarify two terms used in this paper: "undue stress upon the heat dissipating mechanisms" and "negligible increase in heat production." Both of these concepts involve a distinction between mathematically real but physiologically insignificant increments of body heat. Healthy young men fully trained and acclimatized can perform physical labor in the heat in a physiological steady state provided water supply is unlimited

and the degree of exercise is not too severe. The body's mechanisms for maintaining heat balance are so efficient that this same steady state can be maintained under many conditions even when an increase is made in the amount of heat to be dissipated, provided the increase is small. We consider stresses upon the mechanisms for dissipating heat to be insignificant and increases in heat production to be negligible when they do not measurably affect the ability of a subject to maintain a given steady state.

The data in table 3 lead to three main conclusions. First, all subjects showed an improvement in performance during the first two or three periods. We believe this to be the residual acclimatization process described by Robinson, Turrell, Belding and Horvath ('43). These authors distinguish an initial period of about 7 days in which acclimatization to hot environments is quite rapid followed by a long-continued period of much slower acclimatization. All of our subjects had completed the initial period of rapid acclimatization before this experiment began. Second, subjects J.P. and Control exhibited a decline in performance during the last one or two dietary periods. We attribute this to the colds which were previously mentioned as affecting physical fitness in temperate environments. Third, there were no changes in performance at either 95°F. and 85% humidity or 110°F. and 25% humidity which are attributable to dietary protein level. In other words dietary protein may be maintained at any level between 75 and 150 gm. per day for periods of at least 6 weeks without affecting performance in a physical test which may increase the pulse rate by 60 or more beats per minute and raise the rectal temperature by 3 or more degrees Fahrenheit. This finding does not support the recommendation commonly made (Lusk, '31, p. 456) that meat be avoided in hot weather. Similarly Stefansson ('35-36) does not agree with this recommendation since he observed no unpleasant symptoms resulting from an all meat diet during a New York summer.

It has been alleged that increasing the dietary protein level significantly increases the loss of water in sweat. Lusk ('31, p. 409) considers an experiment of Rubner's in which the heat production and avenues of heat loss were studied in a man performing 850 Cal. of work in one day at 20°C. When the subject ate a diet of meat, more of the extra heat production due to work was lost by increased activity of the sweat glands than when he ate cane sugar only. This led Lusk to the conclusion that "a high protein dietary is therefore contra-indicated in athletic contests, especially when the weather is hot and humid." In the present experiments marching under standard conditions of work

in the hot, moist environment led to almost the same hourly loss of sweat independently of dietary period. The averages of the three subjects per hour of marching were: high protein period, 1.19 liters (twenty-two experiments); low protein period, 1.27 liter (twelve experiments). These results are different from Rubner's, and the reasons are made clear by examining Rubner's data. In the first place, his diets were artificial, employing on the one hand cane sugar and on the other meat. In daily life, as in our experiments, diets usually range from 50 to 200 gm. of protein per day. In the second place, the extra loss of water due to protein was, in Rubner's experiment, only 0.6 liters a day. This is an insignificant stress to the body when one considers that a normal, healthy young man is able to work day after day losing 10 or more liters of sweat. Only under conditions when water is strictly limited would this extra loss of sweat due to dietary protein become important.

Observations on respiratory exchange

We shall discuss separately the metabolism while reclining, standing and walking as it was affected by three factors: first, long-term changes due to prolonged subsistence upon the various levels of dietary protein; second, changes during individual days caused by ingestion of food; third, changes due to environment independent of diet. The full data are presented in table 4, the individual values for the three subjects being averaged for the sake of simplicity and clarity. It should be stressed initially that these experiments were not planned to demonstrate the relative specific dynamic action of isocaloric amounts of foodstuffs, but were intended to simulate normal daily life. The subjects, therefore, ate to satisfy hunger, being restricted in the proportions but not the quantity of foodstuffs eaten. Thereby are brought out certain practical points which compensate for the increased variability characterizing data collected under these conditions.

Reclining metabolism. In the present experiment the difference in protein content between the high and low protein diets (which may probably be considered as extremes for normal life) was about 400 Cal. per day. We may assume that the specific dynamic action of this increment is about 100 Cal. which is distributed throughout the 24 hours and probably amounts to not more than 6 Cal. in any 1 hour. Therefore a man with a basal metabolic rate of 1,800 Cal. per day is unlikely ever to increase that rate by more than 5% in changing from a low protein diet to a high one. These expectations are borne out by the data in table 4A. The basal metabolic rate during the high protein period was

1 to 2 Cal./m²/hr. above that during the low protein period. This was 3 to 5% of the total basal rate, a negligible amount from the standpoint of heat dissipation. A second point is that meals whether high, low or normal in protein content led to about the same increase in reclining metabolism, between 8 and 13 Cal./m²/hr. Finally, table 4A shows no consistent effects of environment on total reclining metabolism.

TABLE 4

*Metabolism as affected by type of diet, by individual meals and by environments.
(Average of three subjects.)*

CATEGORY OF INTEREST	HIGH PROTEIN DIET		LOW PROTEIN DIET		NORMAL DIET	
	Fasting	After 2 meals	Fasting	After 2 meals	Fasting	After 2 meals
(A) RECLINING						
Temperate conditions (75°F., 50% relative humidity)						
Metabolism (Cal./m ² /hr.)	39	48	38	46	36	49
Non-protein R.Q.	0.77	0.84	0.80	0.88	0.76	0.87
Pulse (beats/min.)	57	62	57	61	54	64
Rectal temp. (°F.)	98.0	98.4	98.2	98.6
Moist hot conditions (95°F., 85% relative humidity)						
Metabolism (Cal./m ² /hr.)	39	47	37	48	40	49
Non-protein R.Q.	0.83	0.89	0.82	0.87	0.84	0.91
Pulse (beats/min.)	67	72	63	70	61	71
Rectal temp. (°F.)	98.4	99.1	98.5	99.1	98.5	99.1
(B) STANDING						
Temperate conditions (75°F., 50% relative humidity)						
Metabolism (Cal./kg./hr.)	1.15	1.39	1.15	1.33	1.14	1.41
Non-protein R.Q.	0.82	0.87	0.84	0.87	0.81	0.86
Pulse (beats/min.)	74	83	76	79	74	74
Rectal temp. (°F.)	98.4	99.0	98.5	98.9
Moist hot conditions (95°F., 85% relative humidity)						
Metabolism (Cal./kg./hr.)	1.26	1.44	1.20	1.39	1.17	1.48
Non-protein R.Q.	0.88	0.85	0.80	0.87	0.78	0.89
Pulse (beats/min.)	90	91	94	93	84	94
Rectal temp. (°F.)	98.9	99.3	99.1	99.7	99.0	99.5
(C) MARCHING						
Temperate conditions (75°F., 50% relative humidity), speed 3.5 m.p.h., grade 8.6%						
Metabolism (Cal./kg./hr.)	7.80	7.96	7.48	7.67	7.36	7.54
Non-protein R.Q.	0.86	0.90	0.89	0.93	0.87	0.92
Pulse (beats/min.)	125	125	125	123	122	119
Rectal temp. (°F.)	99.4	100.0	99.5	100.1
Moist hot conditions (95°F., 85% relative humidity), speed 3.5 m.p.h., grade 2.5%						
Metabolism (Cal./kg./hr.)	5.09	5.24	4.95	5.13	5.22	5.34
Non-protein R.Q.	0.86	0.88	0.87	0.91	0.83	0.91
Pulse (beats/min.)	124	127	124	121	122	120
Rectal temp. (°F.)	99.9	100.4	100.0	100.6	99.7	100.0

Standing metabolism. Table 4B shows the average standing metabolism for the three subjects. It is expressed in terms of Cal./kg. body weight/hr. since standing metabolism is probably more nearly proportional to body weight than to surface area. The practical conclusions to be drawn from the table are similar to those in the case of reclining. First, the difference between the fasting metabolic rates during the high protein and during the low protein period was small, not exceeding 5%. Second, the post-prandial rise in metabolism varied from 0.18 to 0.31 Cal./kg./hr., being lowest in the low protein period. Third, there was a consistent small effect of environment on total standing metabolism, which was always slightly higher in hot than in temperate environments, regardless of diet or of meals. The difference was at the most 7 Cal. per hour for a 70 kg. man.

Marching metabolism. It will be recalled that in order to impose comparable stresses upon the mechanisms for heat loss, a lower grade of work was employed in the hot, moist room, where it was equivalent in severity to marching at 3.5 m.p.h. on the level with a 40-pound pack, a standard U.S. Army pace. Consideration of table 4C leads to two chief conclusions. First, the overall diminution in metabolism during work in changing from a high protein to a low protein diet amounted at most to 0.3 Cal./kg./hr. This constituted a decrease in the marching metabolic rate of only about 4%. It cannot be said that one is taxing the heat dissipating mechanism of the body when one increases a moderate metabolic rate by 4%. This conclusion is borne out by the actual performance of our subjects. Under environmental conditions which were extremely severe they performed at least as well in the high protein period as they did in the low protein period. The second conclusion is that regardless of protein content of the meals the post-prandial rise in metabolism never exceeded 0.2 Cal./kg./hr. and constituted a negligible heat load according to our criteria.

Non-protein R.Q. Three consistent changes of theoretical interest were noted in non-protein R.Q. First, under both environmental conditions and in all three types of activity there was a post-prandial increase in the non-protein R.Q. of the same magnitude in all three dietary periods. Second, in the reclining state the non-protein R.Q. was higher under moist hot conditions than under temperate conditions. Third, during marching the non-protein R.Q. tended to be lower under moist hot conditions than under temperate conditions. When "partition of nutrients" was calculated by the usual methods, the "percentage of metabolism due to carbohydrate" showed the same changes as the non-protein R.Q.

GENERAL DISCUSSION

The results of the present experiments have some bearing on certain practical aspects of human nutrition. It would appear that for periods of at least 6 weeks dietary protein level may vary widely (from 75 to 150 gm. a day) without affecting physical fitness for intermittent work either under temperate or under hot conditions. Since a high protein diet is preferred by most workmen, it should not be prohibited on the basis of effects on physical fitness or heat dissipation. However, in the special case where water supply is limited, as in a lifeboat, a high protein diet is contra-indicated for two possible reasons. First, the increase in waste nitrogenous products demands extra water for their excretion. Second, the specific dynamic action of protein presumably demands a small increase in perspiration. Neither of these two factors is of any practical significance under ordinary circumstances.

SUMMARY

1. The effect of variation in the level of dietary protein upon the physical fitness and metabolism of three subjects was studied under both temperate and tropical conditions while reclining, standing and marching.

2. The urinary nitrogen excretion in grams per day averaged 18.5 during the high protein period, 9.5 during the low protein period and 12.9 and 13.5 during the normal periods before and after the experiment, respectively. There were minor changes in body weight with a maximum during the high protein period. The plasma protein level showed no significant changes.

3. Physical fitness under temperate conditions showed no changes attributable to dietary protein level.

4. Performance of work in both hot, dry and hot, moist environments showed no changes attributable to dietary protein level. In both cases, however, improvements due to training and acclimatization were observed.

5. Metabolism while reclining and while standing was not significantly different in the high and low protein periods. Metabolism while marching was slightly lower in the low protein period. However, as judged by actual performance in the heat this was a physiologically insignificant change.

6. It is concluded that even though protein does have a high specific dynamic action, the theoretical objections heretofore raised against a high protein diet in hot environments are unjustified under the condi-

tions of our observations. Protein intake may vary widely from 75 to 150 gm. daily without effect upon performance of intermittent work in the heat.

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STUDIES ON THE COMPARATIVE NUTRITIVE VALUE OF FATS

IV. THE NEGATIVE EFFECT OF DIFFERENT FATS ON FERTILITY AND LACTATION IN THE RAT¹

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The dietary requirement necessary to promote lactation adequate for the growth of the new-born rat is more stringent than that required for the growth of older animals or for reproduction. Lactation thus provides a more exacting criterion for the evaluation of the adequacy of a diet than the usual growth method.

Sure ('24a, b) demonstrated that diets satisfactory for growth and reproduction are in some cases inadequate for lactation. A larger intake of the B complex is required for lactation (Sure, '27) and possibly there is a specific component called "B_x", essential for milk production (Sure, '41a). Para-amino benzoic acid was shown to be a component of this factor, a finding which has recently been confirmed (Sure, '43); it was also demonstrated that inositol has an injurious effect on lactation which is counteracted by para-amino benzoic acid. Diets containing 18% of casein as the protein were unsatisfactory unless supplemented with cystine (Sure, '41b). Sure found that neither butter fat, lard, hydrogenated cottonseed oil, olive oil nor wheat germ oil was able to promote satisfactory lactation on an otherwise inadequate diet.

The present tests are an extension of earlier investigations (Deuel et al., '44) where comparisons were made of the growth of young rats on diets of mineralized vitamin-fortified skimmed milk powder containing butter and on similar diets containing corn, cottonseed, olive, peanut or soybean oil, or a margarine in place of the butter.

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In the present study, the effectiveness of such diets in supporting pregnancy and lactation has been followed in rats maintained on these diets from the time of weaning (21 days of age).

EXPERIMENTAL

Three series of experiments consisting of 207 individual tests were carried out. The rats in the first series (series II in Deuel et al., '44a) were bred at 133 days of age after having received the different diets for 16 weeks; those in the second series (series III in the earlier report) were mated at 105 days of age after having received the various diets for 12 weeks. The third series represents some of the rats of our present second series which were rebred 4 weeks after the birth of their first litter. In all cases females were mated with males which had received the same diets. In the first two series, the litters were reduced to seven animals at 3 days of age. Because of the marked effect of the size of the litter on rate of growth, we have averaged the weights of those rats only as long as the litters were maintained at seven animals.

In series I the weights were recorded only at 3 days and 21 days of age; in series II the weights are reported at 3, 7, 14, and 21 days. The litters in series III were reduced to two female rats at 3 days. They were weighed at 3, 7, and 14 days of age at which time they were weaned.

No flavoring was added to the diets in series I. However, after the demonstration in other experiments that flavor plays an important role in food consumption (Deuel and Movitt, '44b), diacetyl² or butter flavor³ was added to the diets employed in series II and III. Other experimental conditions were similar to those in the earlier tests.

A statistical evaluation of the mean weight is applied only to the 21-day weights in series II and to the weights at 14 days in the third series. This is because the average values given for the weights at the earlier periods and for part of the litters at 21 days in series I are based on the litter weight rather than on the weight of the individual rats.

RESULTS AND DISCUSSION

The results of the various experiments are summarized in table 1.

On diets composed of mineralized skimmed milk powder, and a fat fortified with the fat-soluble vitamins essential for the rat, successful

² Obtained from Larkin and Co., Buffalo, New York.

³ "B.F.A." obtained from Verley Products Corporation, 1621 Carroll Avenue, Chicago, Illinois.

TABLE 1

The average weight of rats at several periods before weaning when the mothers had been raised and were maintained on diets of skimmed milk powder and different fats.¹

DIET ^a	FE- MALES BRED	LITTERS		NO. OF RATS IN LITTER AT BIRTH	WEIGHT OF RATS ^b				ANI- MALS DIED
		Born	Des- troyed		3 days	7 days	14 days	21 days ^d	
Series I									
I	9	9	1	9.5	gm. 7.67	gm.	gm.	gm. 31.0(42)	1
II	7	6	0	9.8	7.60			29.6(42)	0
III	6	6	0	7.8	7.96			30.4(14)	2
IV	10	10	1	6.0	6.95			25.9(14)	8
V	4	4	0	10.2	8.49			38.6(28)	0
VI	9	8	0	8.6	7.37			27.3(21)	2
VII	7	7	0	7.3	7.07			32.7(42)	0
Series II									
Ia	14	14	0	9.6	7.41	13.4(77)	24.6(56)	35.8±0.6(42)	22
II	16	14	1	8.9	7.62	13.2(77)	22.8(70)	34.9±0.7(70)	1
III	15	14	1	9.7	8.13	12.8(84)	22.9(84)	34.9±0.5(84)	0
IVa	15	15	1	9.3	7.83	13.6(98)	22.0(98)	36.1±0.5(98)	0
V	12	11	2	7.6	7.53	11.8(49)	21.7(49)	33.0±0.9(42)	1
VI	15	15	1	7.8	7.53	12.4(63)	23.4(63)	35.7±0.5(63)	2
VII	15	14	1	7.6	7.47	12.6(63)	22.8(63)	35.8±0.6(56)	1
Series III									
Ia	6	6	0	7.3	8.19	14.8	31.6±1.1 (12)		0
II	8	8	0	8.8	7.92	14.1	28.7±1.2 (16)		0
III	10	10	0	9.2	7.93	12.7	25.7±1.2 (20)		0
IVa	10	8	0	6.3	8.56	13.6	27.1±1.1 (10)		0
V	5	3	0	5.8	7.19	9.5	20.8 ^e (4)		0
VI	8	8	0	7.9	8.68	14.9	30.1±1.7 (16)		0
VII	6	5	0	8.0	9.60	15.0	31.0±0.9 (10)		0

The figures in parentheses represent the number of animals on which the averages are based.

¹ The diets had the following composition: Mineralized skimmed milk powder 70.6%, the added minerals per 100 lbs. being $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 3.455 gm., $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 3.340 gm., $\text{FeC}_2\text{H}_3\text{O}_7 \cdot 3\text{H}_2\text{O}$, 45.557 gm.; a butter fat, a margarine fat, or vegetable oils supplemented with vitamins and flavor as indicated below per 1000 gm. fat (in series II and III where a whole butter or a whole margarine was used, these were increased so that the same amount of fat was present) 29.4%. The supplements in the fat were: vitamin A—carotene (40% of total A) 8 mg., vitamin A concentrate 133 mg., vitamin D—viosterol 801 mg., vitamin E and tocopherol 10 mg., diacetyl or commercial butter flavor 4 mg.

² The diets containing the various fats are designated as follows: I, butter fat; Ia, whole butter; II, corn oil; III, cottonseed oil; IV, a margarine fat; IVa, a whole margarine; V, olive oil; VI, peanut oil; VII, soybean oil.

³ The average weight at 3 days is the mean weight of all rats. The average values at 7, 14 and 21 days in series I and II are for rats only in those litters where there were seven rats. In series III, the weight at 7 and 14 days are for two in a litter.

⁴ Including the standard error of the mean calculated as follows:

$\sqrt{\frac{ed^2}{n}} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

⁵ Too few experiments for statistical evaluation.

pregnancy ensued irrespective of whether the fat added was a butter, a margarine, or corn, cottonseed, olive, peanut or soybean oil. That the various diets are adequate for fertility in both males and females is indicated by the fact that over 94% of the females cast litters. If fat per se is required for fertility and pregnancy, its requirement may be equally well satisfied by the various vegetable fats as by butter.

From the standpoint of lactation, as judged by the survival of the litters and the weight of the young at weaning time, the different fats were also equally effective. No statistical differences were found in series II or series III where the groups were sufficiently large for a statistical evaluation. Although such an analysis was applied only to the 21- and 14-day weights respectively in these two groups, it is evident from the inspection of the average weight at 3, 7, and 14 days that these values for the rats on the various diets are all within experimental error. A possible exception is the olive oil group in series III which would appear to be definitely lower than the other groups. On the other hand, the rats receiving the olive oil diet in series I have a considerably higher average weight than the rats on the diets containing the other fats. This seeming discrepancy may be because the number of rats in series III which received the olive oil is too small on which to base any conclusions (only two litters). It might also be due to the inferior grade of olive oil which was used in the third series as contrasted with the first and second series when a more satisfactory sample was available. In series II, values of this group are within the experimental error of the other groups.

With the exception of the animals receiving the olive oil diet, the weight of the rats in series III at 14 days was 20 to 30% higher than in the second series. This is probably to be ascribed to the fact that the litters were reduced to two rats in the former case although there is also the possibility that it may partly be due to the fact that the rats in the third series were from second litters which are usually somewhat larger than those from the first litter.

The reason for the large mortality in the group receiving the butter diet in series II is unknown. This was not observed in the first or third series; moreover, growth of the mothers which had been started on this diet at 21 days of age had previously been shown to be satisfactory (Deuel et al., '44a).

SUMMARY

No differences were found in the fertility of male or female rats which from 21 days of age had received diets of mineralized skimmed milk powder fortified with the necessary fat-soluble vitamins and vari-

ous fats irrespective of whether the lipid was butter, a margarine, corn, cottonseed, olive, peanut, or soybean oil. Moreover, such diets were equally efficient in promoting lactation as judged by the weights of the rats when weaned at 14 or 21 days of age.

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